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Analysis of HOXA5 expression and function in development of the central nervous system

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NOTE TO USERS

Analysis of HOXA5 expression and function in development of the central nervous system

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This is to certify that the doctoral dissertation of

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has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

To Luka, Aleksandra, and my parents

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CHAPTER I. General Introduction

Thesis Organization

This thesis is organized in five chapters and an appendix. Chapter I provides a general introduction for the thesis. Chapter II is a literature review including background information and context for the original work presented in Chapters III and IV and the Appendix. Chapter III is the first author manuscript in press in *Gene Expression Patterns* (Joksimovic et al., 2005). Chapter IV is the first author manuscript to be submitted to *Developmental Biology* (Joksimovic et al., 2005). Chapter V is a summary of work presented herein and general conclusions. The thesis author also contributed to the characterization of the *Hoxa5SV2* mouse line that led to a co-authored publication in *Developmental Brain Research* (Krieger et al, 2004) and to a co-authored manuscript to be resubmitted after a revision to *Developmental Brain Research* (Abbott et al., 2005). The Appendix is divided into three parts: the Appendix I is a detailed description of development of HOXA5-specific antibodies; a substantial work toward a collaborative project and co-authored publication (Coulombe et al., 2005; manuscript in preparation) is presented in the Appendix II; the Appendix III is a discussion of the limitation of use of the yeast-one hybrid technology to identify a *Hoxa5* enhancer-binding protein.

General Introduction

Development of multicellular organisms is a complex process encompassing differential expression of the genetic information in a precise spatial and temporal manner.

Hox genes, which encode transcription factors, have critically important functions in the regulation of morphogenesis and in the specification of regional identity along the embryonic axis. Expression of *Hox* genes is spatially and temporally specific, and this property is critical to their function. Mutations in *Hox* genes cause homeotic transformations in the skeleton and defects in organogenesis that impair viability.

In mice, one of the *Hox* genes, *Hoxa5,* is expressed during embryogenesis in the central nervous system (CNS) and in various mesoderm-derived structures such as developing respiratory tract, prevertebrae (pv), and gut. A disruption of the *Hoxa5* gene is associated with a high perinatal mortality rate and alterations in development of several structures with mesodermal origin confirming the essential role of *Hoxa5* during mouse development. However, neither behavioral nor molecular CNS phenotypes have been described for *Hoxa5* deficient animals.

Transcription of the *Hoxa5* gene leads to a complex pattern of multiple mRNAs that are differentially expressed in specific mesoderm-derived structures affected by the *Hoxa5* mutation, and in the most anterior domain of expression in the CNS, in contrast to indistinguishable patterns seen in the posterior CNS. Nonetheless, the functional significance of any pattern of mRNAs must be confirmed by correlating the presence of the protein(s) and RNAs. To do so, we generated specific HOXA5 antibodies, analyzed the protein localization during embryogenesis and showed that the HOXA5 protein expression profile is concordant with the expression pattern of one of the *Hoxa5* transcripts shown to be essential for proper development of mesoderm-derived structures. Moreover, we detected a highly dynamic and region-specific expression pattern in the CNS. This work is presented in the Chapter III and the Appendix II. Consequently, our findings presented in the Chapter III led us to investigate

the intricate expression pattern seen in the CNS in an effort to determine the function of HOXA5 in this complex system. With a focus on understanding the combinatorial transcriptional code in cellular specification of the caudal hindbrain, Chapter IV provides, to date, the first evidence for the role of any *Hox5* paralogue in proper development of this structure. Details of this work are presented in Chapter IV. Additionally, the yeast-one hybrid system was used to gain a better understanding of *Hoxa5* regulation in the attempt to identify cDNAs encoding *Hoxa5* enhancer-binding protein. Unfortunately, although different baits and several yeast strains were tested, the system appeared to be unsuitable for directly cloning the cDNA and subsequently identifying a *Hoxa5* enhancer-binding protein. This work is summarized in the Appendix III.

References

Abbott, M.A., **Joksimovic, M.,** Tuggle, C.K., 2005. Ectopic HOXA5 expression results in abnormal differentiation, migration and p53-independent cell death of superficial dorsal horn neurons (to be resubmitted after a revision to Developmental Brain Research)

Coulombe, Y., Lemieux, M., Moreau, J., Tabariès, S., Larochelle, C., **Joksimovic, M.,** Tuggle, C.K., Aubin J., Jeannotte, L., 2005. Different promoters and alternative splicing: transcriptional complexity at the murine *Hoxa5* locus (manuscript in preparation)

Joksimovic, M., Jeannotte, L., Tuggle, C.K., 2005. Dynamic expression of murine HOXA5 protein in the central nervous system (in press; Gene Expression Patterns)

Joksimovic, M., Abbott, M.A., Tuggle, C.K., 2005. Role of murine HOXA5 in development of the inferior olivary nucleus (to be submitted to Developmental Biology)

Krieger, K.E., Abbott, M.A., **Joksimovic, M.,** Lueth, P.A., Sonea, I.M., Jeannotte, L., Tuggle, C.K., 2004. Transgenic mice ectopically expressing HOXA5 in the dorsal spinal cord show structural defects of the cervical spinal cord along with sensory and motor defects of the forelimb. Brain Res. Dev. Brain Res. 150, 125-139.

CHAPTER II. Literature Review

Anatomy of the medulla oblongata and spinal cord

The medulla oblongata is a part of the central nervous system (CNS) that develops from the posterior hindbrain during embryogenesis. This structure is an important relay center for sensory and motor systems, spanning the area between the midbrain and the spinal cord. Within the medulla oblongata, distinct clusters of neuronal cell bodies (nuclei), located in stereotypic positions along the anterior-posterior (AP) and dorso-ventral (DV) axes, are involved in the perception of multiple sensations, such as proprioception, pain, touch, temperature, balance, taste, and control of respiration and motor activity. Dorso-medially are located the hypoglossal and dorsal vagal nuclei and nucleus of tractus solitaruis (nTS) while the nucleus cuneatus and the nucleus gracilis occupy the dorso-lateral aspect of the medulla. The spinal trigeminal nucleus is situated throughout the lateral region of the medulla whereas the lateral reticular and raphe nuclei span the ventro-lateral and midline region, respectively. The most prominent structure of the adult ventral medulla is the convoluted inferior olivary nucleus (ION), made up of three subdivisions: the medial accessory olive (MAO), dorsal accessory olive (DAO), and principal olive (PO). The DAO contains the dorsal and ventral lamellae joined laterally to form a twisted V-shaped structure (Azzizi et al., 1987). The ION, through climbing fibers, has a massive input to the cerebellum and is involved in motor control and learning memory (Sherrard and Bower, 2002).

The spinal cord is comprised of the white matter and the gray matter. The latter can be divided into a dorsal half receiving sensory information from the periphery and a ventral half innervating body musculature. Within the dorsal spinal cord, prominent neuronal cell

types are interneurons while the ventral spinal cord contains primarily motor neurons and some interneurons. The forelimb (brachial) and hindlimb (lumbar) levels of the spinal cord contain an increased number of motor neurons that are organized into longitudinal columns necessary to transmit motor output to the limb musculature.

Cellular specification of the neural tube

The neural tube forms the brain anteriorly and posteriorly the spinal cord. Early mammalian brain is divided into three primary vesicles: forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon). The rhombencephalon is eventually subdivided into an anterior metencephalon that gives rise to the pons and the cerebellum and a posterior myelencephalon that becomes the medulla oblongata (Gilbert, 2000).

The mature CNS contains different neuronal and glial subtypes generated and specified during development of the neural tube. In recent years, the majority of data regarding a neuronal cell fate determination has been collected from spinal cord studies in chick and mouse. In the dorsal neural tube, progenitor cell types are defined by four non-overlapping expression domains of proneural genes *Math1, Neurogenin1 and 2, and Mash1* which encode basic-helix-loop-helix transcription factors. The expression domains of these proneural genes are maintained and regulated at the transcriptional level by their mutually repressive interactions (Gowan et al., 2001). The progenitor cells eventually generate six classes of early-borne (E10-12) postmitotic dorsal interneurons (dI) that migrate to the deep dorsal horn and towards the ventral horn, and two classes of late-borne (E11-13), postmitotic dI migrating to superficial regions of the dorsal horn (Matise et al., 2002; Gross et al., 2002;

Muller et al., 2002). These classes of dorsal interneurons can be distinguished on the basis of the combinatorial expression of homeodomain transcription factors such as LH2A/B and BARHL1 (dI1), LBX1 (dI4-6), LIM1/2 (dI2, 4, 6), PAX2 (dI4, 6), LMX1B (dI5), ISL1/2 (dI3), BRN3A (dI1, 2, 3, 5), FOXD3 (dI2), RNX/TLX3, and TLXL (dI3, 5)(Gowan et al., 20001; Matise et al., 2002; Gross et al., 2002; Muller et al., 2002). The six classes can be further classified by their dependence on roof plate signaling in class A (dI1–3) neurons that are dependent on, and class B (dI4–6, and two classes of late born neurons) that are independent on roof plate signals (Muller et al., 2002; Lee et al., 2000). Specification of neuronal identity within the dorsal spinal cord is initiated from a primary signaling center - the surface ectoderm. Signals from the surface ectoderm through the bone morphogenetic proteins (BMPs) 2, 4, and 7 can specify the roof-plate cells - the secondary signaling center. Next, this center specifies the dorsal interneuron cell types of the neural tube through activity of BMPs, activin, Wnts, and antagonist of sonic hedghog (Dickinson et al., 1995; Liem et al., 1995; Lee et al., 1998). The differentiation of the ventral cell type is controlled through activity of the floor-plate by the primary signaling center, which is composed of the axial mesoderm and the notochord. The main signaling activities of the notochord and the floor-plate encompass a secreted protein, Sonic hedgehog (SHH)(Patten and Plazcek, 2000) that is required for induction of the majority ventral cell types in *vitro* and *in vivo* (Marti et al., 1995; Ericson et al., 1996) A set of homeodomain transcription factors expressed by neural progenitors act as intermediary factors in the interpretation of graded SHH signaling (Pierani et al., 1999; Ericson et al., 2000; Briscoe et al., 2000) leading to generation of five classes of postmitotic ventral neurons (V0, V1, V2, motor neurons, and V3). Each class exclusively

expresses unique transcription factor: EVX1/2 (V0), EN1 (V1), CHX10 (V2), ISL1 (MN), and SIM1 (V3)(Goulding et al., 2002).

Recent studies revealed a combinatorial transcriptional code for some of hindbrain nuclei. In the dorsal hindbrain, the developing nTS is marked by expression of LMX1B, PHOX2B and RNX. Moreover, PHOX2B and RNX are essential for the formation of nTS and relay visceral sensory neurons (Qian et al., 2001; Dauger et al., 2003). The LMX1B transcription factor is also expressed and crucial for proper development of raphe nucleus and the expression of a neurotransmitter serotonin (Ding et al., 2003). The other transcription factors such as BARHL1 and PAX6 are necessary for proper formation of the lateral reticular and external cuneate nuclei (Engelkamp et al., 1999; Li et al, 2004). The BRN3A and BRN3B transcription factors are expressed in the ION during development. *Brn3a* deficient animals display defective suckling and uncoordinated limb and trunk movements that lead to early postnatal lethality indicating that the *Brn3a* gene has important developmental impact for the somatosensory system and hindbrain nuclei involved in motor control (Xiang et al., 1996). Despite accumulated data and in contrast to the spinal cord studies (Gowan et al., 2001; Matise et al., 2002; Gross et al., 2002; Muller et al., 2002), no comprehensive analysis has been performed to determine the combinatorial transcriptional code in the hindbrain and its relationship to the code detected within the spinal cord.

Introduction to *Hox* genes and their role in hindbrain and spinal cord development

Mammalian *Hox* genes constitute a family of 39 genes clustered in four independent groups (*Hoxa-d*) belonging to the *Drosophila Antennapedia* homeodomain class (Krumlauf, 1994). Based upon protein sequence similarity *Hox* genes are grouped into 13 paralog

subfamilies, the members of which are represented once in almost every complex. The most apparent characteristic of the *Hox* complex is a collinear relationship between the relative order of the *Hox* genes on the chromosome, their precise domain of expression along the AP axis, and their response time to retinoic acid treatment: the 3'-most genes are most responsive to retinoic acid, are expressed earlier during embryogenesis, and are in more anterior domains than their 5' counterparts (Krumlauf, 1994). The organization of the constituent genes in each *Hox* complex is essential for the precise spatio-temporal regulation and the function of each gene and subsequently for the correct patterning of the embryo (van der Hoeven et al., 1996; Gould et al., 1997; Mann et al., 1997; Kondo et al., 1998). *Hox* genes encode helix-turn-helix transcriptional regulatory factors that share a highly conserved 61-amino acid DNA-binding domain termed a homeodomain. Most of the characterized homeodomains specifically recognize a DNA sequence containing a 5'-TAAT-3' core but, despite having similar DNA-binding specificities *in vitro*, individual HOX proteins confer extremely specific regulatory actions *in vivo*. This differential selectivity is assumed to be achieved via the cooperative DNA binding of HOX proteins with cofactors, such as the PBX/EXD, Maf oncoprotein, PBX/MEIS dimer (Mann and Chan, 1996; Kataoka et al., 2001, Shanmugam et al., 1999) or other HOX proteins (Sanlioglu et al., 1998).

During early development, the hindbrain is subdivided into seven neuromeric segments called rhombomeres (r). Each rhombomere has a distinct developmental fate and will eventually generate sensory and motor nuclei. Hindbrain segmentation is a transient process occurring during the second half of the first month in humans, between embryonic day (E)8 and E12 in mice, and between E1 and E4 in chicks (Borday et al., 2003). *Hox* gene paralog 1-4 are expressed in the early developing hindbrain and have overlapping *RNA*

expression domains with a defined anterior limit that can be associated with the position of the gene in the cluster. *Hox4* paralogs *(a, b,* and *d)* are expressed up to r6/r7 boundary while *Hox3* paralogs *(a, b,* and *d)* have the anterior boundary of expression at r4/r5 junction. However, *Hoxa1* and *Hoxb1* are expressed up to r3/r4 boundary while *Hoxa2* and *Hoxb2* show anterior limit of expression at r1/r2 and r2/r3, respectively breaking a rule of colinearity. In addition, *Hoxa2* is the only *Hox* gene expressed in r2. As a consequence, each rhombomere has the unique combination of a particular level of *Hox* gene expression often referred to as '*Hox*' code that could specify the positional identity and cell fates in the developing hindbrain (Hunt, 1991). For instance, targeted disruption of the murine *Hoxa1* results in severe defects of the hindbrain organization and related cranial ganglia and nerves. Rhombomere 5 character is missing and motor neurons, which would normally be present in the missing rhombomere, appear to be respecified and migrate into neighboring rhombomeres (Carpenter et al., 1993). In addition, the sixth cranial nerve (abducens) is absent in these mutants (Mark et al., 1993). *Hoxb1* is coexpressed with *Hoxa1, Hoxa2,* and *Hoxb2* in r4, which produces facial branchiomotor neurons. *Hoxb1* mutant mice display a change in r4 identity and facial branchiomotor neurons fail to migrate into their proper positions (Studer et al., 1996). However, *Hoxb1* mutant motor neurons differentiate in r4 suggesting that *Hoxb1* is not required to initiate motor neuron generation. Mice with a compound mutation of both *Hoxa1* and *Hoxb1* exhibit a more severe phenotype than each single mutant, supporting the '*Hox*' code concept. On the other hand, the activity of a single *Hox* gene can specify distinct neuronal subtypes. An ectopic expression of *Hoxb2* or *Hoxa1,* anterior to their normal expression domains, in r1 *in vivo* results in the generation of ectopic facial and trigeminal branchiomotor neurons, respectively, in the region that never produces

any motor neurons indicating that individual *Hox* genes can be sufficient to induce some neuronal subtypes (Jungbluth et al., 1999). Therefore, it appears that *Hox* genes can function not only through individual activities but also through their combinatorial mode of action. In addition, the combined effects of compensation, synergy and functional redundancy often obscure individual *Hox* gene function (Morrison, 1998; Chen et al., 1998; Barrow et al., 2000). An initial '*Hox*' code may also be operating later in development; ectopic *Hoxa1*, whose expression is transient in the hindbrain, is sufficient to give a novel functional neural circuit leading to alteration of the rhythm-generating network controlling respiration at birth (del Toro et al., 2001). It is known that *Hox5* paralog genes are not expressed in early developing hindbrain. However, *Hoxb5* undergoes a retinoic acid-dependent neuronal expansion into the caudal hindbrain between E10.5 and E11.5 (Oosterveen et al., 2003) suggesting that the other two *Hox5* paralog genes (*Hoxa5* and *Hoxc5*) may show similar temporal trend and possibly function in further development of the caudal hindbrain.

In the spinal cord, *Hoxc8* and *Hoxd10* are critical for the normal development of motoneurons that control forelimb and hindlimb movements, respectively (Carpenter et al., 1997; Tiret et al., 1998). *Hoxb5* deficient mice display an altered trajectory of spinal nerves (Rancourt et al., 1995). Mice harboring a mutation in *Hoxa10* gene show an anterior homeotic transformation of the first lumbar nerve that appears as an intercostal, the last thoracic nerve (Rijli et al., 1995). Gain-of-function studies also shed a light on the important function of *Hox* genes in the spinal cord development. In one study, a normal degeneration of the most anterior dorsal root ganglion, was prevented by ectopic expression of *Hoxb8* (Fanarraga et al, 1997). In addition, members of the *Hoxc* cluster have been shown to play a crucial role in determining motor neuron columnar identity domains set up by cross-

repressive interactions of *Hoxc6* and *Hoxc9* genes (Dasen et al., 2003). Taken together, these studies provide considerable evidence for an essential role of *Hox* genes in CNS development.

Function and the expression pattern of murine *Hoxa5*

A phenotypical analysis of the *Hoxa5* mutant mice has shown the importance of this gene in the survival of newborn animals and in the specification of axial identity. *Hoxa5* contributes to the proper specification of the cervical and upper thoracic region of the skeleton and to the correct formation of the pectoral girdle (Jeannotte et al., 1993; Aubin et al., 1998; 2002a). The high rate of perinatal lethality of *Hoxa5* deficient animals was attributed to the impaired development of the respiratory tract (Aubin et al., 1997). In addition, *Hoxa5* is involved in the functional maturation of the gut and specification of the gastric epithelium (Aubin et al., 1999; 2002b). Finally, the *Hoxa5* mutation alters thyroid development causing transient hypothyroidism (Meunier et al., 2003). These results suggested that the *Hoxa5* gene had a significant role in the development and specification of the cervical and upper thoracic region of the skeleton and other cervico-thoracic structures indicating the importance of the *Hoxa5* gene for the proper patterning of the embryo (Jeannotte et al., 1993; Aubin et al., 1998). Although structural deficits of the cervical spinal cord, sensory and motor defects involving the forelimb, were reported in mice ectopically expressing HOXA5 protein in the dorsal spinal cord (Krieger et al., 2004), no CNS phenotype has been described for *Hoxa5* deficient animals.

Transcription of the *Hoxa5* gene leads to a complex pattern of multiple mRNAs during embryogenesis and in adult tissues (Odenwald et al., 1987; Murphy et al., 1988;

Zakany et al., 1988; Jeannotte et al., 1993). Four *Hoxa5* transcripts, short (1.8kb) and three larger ones (4.9, 9.5 and 11 kb) are all transcribed from the *Hoxa5* locus (Jeannotte et al., 1993). The proximal (1.8 kb) transcript is the most abundant poly-A transcript in the embryo, and corresponds to the 2 exons of *Hoxa5* gene (Zakany et al., 1988). A presumptive transcription initiation site of the 9.5 and 11 kb transcripts appear to be in the *Hoxa6-Hoxa7* intergenic region. The transcription start site of the 5.0 kb transcript could be identical or in the vicinity of that of the 9.5 and 11 kb transcripts. The difference in size between the 9.5 and 11 kb transcripts is explained by the 1.5 kb *Hoxa6* intron, which is only present in the 11 kb transcript, and that an intron of at least 3 kb in size is located within or 5' to the *Hoxa6* exon 1 (L. Jeannotte; unpublished data). RNase protection and primer extension analyses have localized the 5' end of the proximal transcript at a position -74 and -44 relative to the ATG codon corresponding to the predicted HOXA5 protein of 270 amino acids (Zakany et al., 1988; Murphy et al., 1988). In addition, it appears that all four transcripts end at the same site based upon 3'RACE analyses (L. Jeannotte; unpublished data). The presence of long and interspersed transcripts also strengthens the significance of the *Hox* cluster organization for the correct expression of *Hox* genes. It is not known if these alternative transcripts encode different functional forms of the HOXA5 protein. However, only the larger transcripts contain an upstream AUG codon that could potentially extend the HOXA5 open reading frame by an additional 110 amino acids.

Initially, RNA *in situ* hybridization analysis using probes that recognized all four transcripts localized *Hoxa5* expression in the mesenchymal component of several structures, the spinal cord, and the prevertebral column (Dony and Gruss, 1987; Gaunt et al., 1990). *In situ* analysis using distinct probes that recognized only the three larger transcripts or all four

transcripts revealed differential expression of *Hoxa5* during embryogenesis (Aubin et al., 1998). However, the presence of the short transcript can be inferred due to a lack of hybridization signal with a probe recognizing larger transcripts only and the presence of a hybridization signal with a probe recognizing all four *Hoxa5* transcripts. The larger transcripts were expressed later and in more posterior structures than the short transcript (Larochelle et al., 1999). The anterior limit of expression of the larger transcripts in the prevertebral column corresponded to prevertebra (pv) 10, while the deduced expression of the short transcript was found between pv3 and pv10 (Aubin et al. 1998). The presence of the short transcript was also deduced in the trachea, lung mesenchyme, and myenteric plexus of the gut (Jeannotte et al., 1993; Aubin et al. 1998; Aubin et al., 1999). Interestingly, an anterior limit of all four *Hoxa5* mRNAs in the neural tube changes between E10.5 and E12.5. The anterior limit of expression was detected within the caudal hindbrain at E12.5 while the limit was closer to the spinal cord junction at E10.5 (Larochelle et al., 1999). Although the *Hoxa5* mutation eliminated expression of the four normal *Hoxa5* transcripts (Jeannotte et al., 1993), only the regions where the expression of the proximal transcript is inferred, such as the respiratory tract, gut and the cervical region of the skeleton, were shown to be affected by the mutation (Aubin et al., 1997; Aubin et al., 1998; Aubin et al., 1999). Thus, it seems likely that the short (1.8 kb) transcript could be the functional form of the *Hoxa5* gene. However, the functional significance of any RNA pattern has to be verified by correlating the presence of the protein(s) and RNAs. It is therefore of great interest to determine if the HOXA5 protein produced from this proximal transcript is confined to the region affected by the *Hoxa5* mutation.

Although there is little data on the relative distribution of *Hox* RNAs versus HOX proteins, the RNA/protein distribution matches several genes such as *Hoxb5* and *Hoxc8* along the antero-posterior axis in the neural tube and paraxial mesoderm, *Hoxa2* in the neural tube, and *Hoxb4* in paraxial mesoderm (Awgulewitsch and Jacobs, 1990; Conlon and Rossant, 1992; Wall et al., 1992; Belting et al., 1998; Sharpe et al., 1998; Hao et al., 1999; Brend et al., 2003) indicating primarily transcriptional control for regulation of these genes. However, in the neural tube, HOXB4 protein is detected only in an anterior subdomain of the region where this gene is transcribed (Brend et al., 2003) suggesting translational control of *Hoxb4* expression. This implies that each *Hox* gene has a region-specific determinative function and may be involved in the regulation of the structural organization of tissues during embryogenesis.

Development of the nucleus of tractus solitarius and inferior olivary nucleus

Nucleus of tractus solitarius (nTS)

Physiological control of the cardiovascular system and respiration involves complex neuronal circuitry. Rhythmic neurons in the ventral medulla produce respiration patterns that are modulated by inputs from other neuronal groups in the brainstem (Blessing 1997). For example, inputs from pulmonary stretch receptors and peripheral chemoreceptors are conveyed via visceral sensory neurons in the medio-dorsally located nTS leading to an increase of respiration rate. On the contrary, inputs from (nor)adrenergic neurons to the respiratory muscles via the ventral hindbrain cause a decrease of respiratory frequency. These neurons are embedded in the nTS in medullary A2C2 catecholaminergic center and express tyrosine hydroxylase (TH), a rate-limiting enzyme in the synthesis of adrenalin and

noradrenalin (Blessing 1997). During development, nTS neurons start migrating from a lateral edge of the dorsal caudal hindbrain at E10.5 and settle in their definitive, medio-dorsal position at E13.5. These neurons coexpress LMX1B and PHOX2B, and RNX and PHOX2B transcription factors in the (Qian et al., 2001; Dauger et al., 2003). The expression of tyrosine hydroxylase is initiated later in development forming the A2C2 catecholaminergic center. Although PHOX2B protein is crucial for formation of the (nor)adrenergic neurons throughout the nervous system, PHOX2B is also expressed in non-(nor)adrenergic structures, indicating that it is required but not sufficient to specify the (nor)adrenergic phenotype (Pattyn et al. 2000). RNX is essential for proper formation of relay visceral sensory neurons in the brainstem. Besides, the development of majority of (nor)adrenergic centers is affected in *Rnx*-deficient mice (Qian et al., 2001).

A disruption of the *Hoxa5* gene results in impaired tracheal and lung development that is likely to be the cause of early postnatal lethality of *Hoxa5*-deficient mice (Aubin et al., 1997). However, surviving *Hoxa5-/-* mutant animals display respiratory adaptations involving a higher frequency of breathing and overall minute ventilation that likely compensates for altered morphogenesis of the respiratory tract (Kinkead et al., 2004). Additionally, potential HOXA5 recognition sites shared across human, mouse, and rat has been reported in the tyrosine hydroxylase promoter (Kessler et al., 2003) and PHOX2 and HOXA5 proteins can interact with the human norepinephrine transporter gene promoter *in vitro* (Kim et al., 2002). However, a CNS phenotype for *Hoxa5* deficient animals or a potential role of HOXA5 in neuronal control of breathing has not been described.

Inferior olivary nucleus (ION)

Several precerebellar nuclei are generated from neuronal populations originating in the rhombic lip, which consists of the upper (rostral) and lower (caudal) lips. The rostral lip produces only cerebellar granule cell precursors (Hallonet et al., 1990). The caudal lip forms the proliferative precerebellar neuroepithelium (pn) (Altman and Bayer, 1987) in the dorsal medulla. Neurons generated by the pn migrate along different paths, to form the precerebellar nuclei, all of which project to the cerebellum (Altman and Bayer, 1987). These nuclei are situated in the pontine and medullary regions of the hindbrain. The five pre-cerebellar nuclei are: the reticulotegmental and pontine nuclei in the pons and the external cuneate, lateral reticular nuclei and inferior olive in the medulla (Altman and Bayer, 1987). Three migration pathways bring cells from the pn to the five nuclei. The posterior extramural stream (pes) cross the midline and settle contralaterally in the external cuneate and lateral reticular nuclei in the medulla (Altman and Bayer, 1987). The anterior extramural stream (aes) goes through several rhombomere boundaries (Marín and Puelles, 1995) before settling ipsilaterally in reticulotegmental and the pontine nuclei. The ION is formed by cells using the third, intramural, migration stream passing through the medullary parenchyma.

In mice, the time of birth of the inferior olive neurons is between E10-11 and they start migrating toward ventral hindbrain at E11-12. A day later, neurons begin to reach the vicinity of the floor plate and settle in their definitive position in the ventral medulla. At E15, inferior olive neurons start reaching the cerebellar parenchyma. At E16, the ION appears as a foliated structure (Bloch-Gallego et al., 1999) while early transient synaptogenesis (P0-P5) and climbing fiber maturation including synaptic stabilization (P7-P15) occur after birth (Sherrard and Bower, 2002). The ION is the sole source of climbing fibers that project to the

contralateral cerebellum. In addition, projections from the inferior olive subnuclei appear to be specific relative to their termination sites in the cerebellum (Sugihara and Shinoda, 2004).

In mice deficient for production of the chemoattractant *netrin-1,* a majority of olivary cells are ectopically located within the migratory stream and fail to reach the floor-plate region (Bloch-Gallego et al., 1999). Another study reported the *Slit* could silence the attractive effects of *netrin-1* and play a role in the proper ventral positioning of the ION by stopping the migration when olivary cell bodies reach the floor-plate (Causeret et al., 2002). Several transcription factors have been reported to be expressed in the ION. BRN3A was detected throughout the rostro-caudal extend of the ION, while BRN3B expression was present in many neurons in the rostral and ventral caudal ION but was not found in the dorsal caudal ION. In *Brn3a* deficient animals BRN3B expression was preserved in the ION whereas the authors reported a decrease in the cell number specific to dorso-caudal ION (Xiang et al., 1996). Although the ION displays rostro-caudal developmental specification, reminiscent of other hindbrain structures dependent on *Hox* function, no data are currently available concerning the expression or function of any *Hox* gene in the inferior olivary complex.

References

Altman, J., Bayer, S., A., 1987. Development of the precerebellar nuclei in the rat: II. The intramural olivary migratory stream and the neurogenetic organization of the inferior olive. J. Comp. Neurol. 257, 490 –512.

Aubin, J., Lemieux, M., Tremblay, M., Bérard, J., Jeannotte, L., 1997. Early postnatal lethality in *Hoxa-5* mutant mice is attributable to respiratory tract defects. Dev. Biol. 192, 432-445.

Aubin, J., Lemieux, M., Tremblay, M., Behringer, R. R. and Jeannotte, L. 1998.

Transcriptional interferences at the *Hoxa4/Hoxa5* locus: Importance of correct *Hoxa5* expression for the proper specification of the axial skeleton. Dev. Dyn. 212, 141-156.

Aubin, J., Chailler, P., Ménard, D., Jeannotte, L., 1999. Loss of *Hoxa5* gene function in mice perturbs intestinal maturation. Am. J. Physiol. 277, C965–C973.

Aubin, J., Lemieux, M., Moreau, J., Lapointe, J., Jeannotte, L., 2002a. Cooperation of *Hoxa5* and *Pax1* genes during formation of the pectoral girdle. Dev. Biol. 244, 96-113.

Aubin, J., Déry, U., Lemieux, M., Chailler, P., Jeannotte L., 2002b. Stomach regional specification requires *Hoxa5*-driven mesenchymal-epithelial signaling. Development 129, 4075-4087.

Awgulewitsch, A., Jacobs, D., 1990. Differential expression of Hox 3.1 protein in subregions of the embryonic and adult spinal cord. Development 108, 411-20.

Azizi, S.A., Woodward, D. J., 1987. Inferior olivary nuclear complex of the rat: morphology and comments on the principles of organization within the olivocerebellar system. J. Comp. Neurol. 263, 467-484.

Barrow, J.R., Stadler, H.S., Capecchi, M.R., 2000. Roles of Hoxa1 and Hoxa2 in patterning the early hindbrain of the mouse. Development 127, 933-944.

Belting, H.G., Shashikant, C.S., Ruddle, F.H., 1998. Multiple phases of expression and regulation of mouse Hoxc8 during early embryogenesis. J. Exp. Zool. 282, 196-222.

Blessing, W.W., 1997. The lower brainstem and body homeostasis. Oxford University Press, New York, NY.

Bloch-Gallego, E., Ezan, F., Tessier-Lavigne, M., Sotelo, C., 1999. Floor plate and netrin-1 are involved in the migration and survival of inferior olivary neurons. J. Neurosci. 19, 4407-4420.

Brend, T., Gilthorpe, J., Summerbell, D., Rigby, P.W., 2003. Multiple levels of transcriptional and post-transcriptional regulation are required to define the domain of Hoxb4 expression. Development 130, 2717-2728.

Briscoe, J., Pierani, A., Jessell, T.M., Ericson, J., 2000. A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101, 435-45

Carpenter, E.M., Goddard, J.M., Chisaka, O., Manley, N.R., Capecchi, M.R., 1993. Loss of Hox-A1 (Hox-1.6) function results in the reorganization of the murine hindbrain. Development 118, 1063-75.

Carpenter, E.M., Goddard, J.M., Davis, A.P., Nguyen, T.P., Capecchi, M.R., 1997. Targeted disruption of Hoxd-10 affects mouse hindlimb development. Development 124, 4505-4514.

Chen, J., Ruley, H.E., 1998. An enhancer element in the EphA2 (Eck) gene sufficient for rhombomere-specific expression is activated by HOXA1 and HOXB1 homeobox proteins. J. Biol. Chem. 273, 24670-24675.

Conlon, R.A., Rossant, J., 1992. Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. Development 116, 357-368.

Causeret, F., Danne, F., Ezan, F., Sotelo, C., Bloch-Gallego, E., 2002. Slit antagonizes netrin-1 attractive effects during the migration of inferior olivary neurons. Dev. Biol. 246, 429-440.

Dasen, J.S., Liu, J-P., Jessell, T.M., 2003. Motor neuron columnar fate imposed by sequential phases of Hox-c activity. Nature 425, 926-933.

Dauger, S., Pattyn, A., Lofaso, F., Gaultier, C., Goridis, C., Gallego, J., Brunet, J-F., 2003. Phox2b controls the development of peripheral chemoreceptors and afferent visceral pathways. Development 130, 6635-6642.

del Toro, E.D., Borday, V., Davenne, M., Neun, R., Rijli, F.M., Champagnat, J., 2001. Generation of a novel functional neuronal circuit in Hoxa1 mutant mice. J. Neurosci. 21, 5637-5642.

Dickinson, M.E., Selleck, M.A., McMahon, A.P., Bronner-Fraser, M., 1995. Dorsalization of the neural tube by the non-neural ectoderm. Development 121, 2099-2106.

Ding, Y.Q., Marklund, U., Yuan, W., Yin, J., Wegman, L., Ericson, J., Deneris, E., Johnson, R.L., Chen, Z.F., 2003. Lmx1b is essential for the development of serotonergic neurons. Nat. Neurosci. 6, 933-938.

Dony, C., Gruss, P., 1987. Specific expression of the *Hox 1.3* homeo box gene in murine embryonic structures originating from or induced by the mesoderm. EMBO J. 6, 2965-2975.

Engelkamp, D., Rashbass, P., Seawright, A., van Heyningen, V., 1999. Role of Pax6 in development of the cerebellar system. Development 126, 3585-3596.

Ericson, J., Morton, S., Kawakami, A., Roelink, H., Jessell, T.M., 1996. Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell 87, 661-673.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T.M., Briscoe, J., 1997. Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell 90, 169-180.

Fanarraga, M.L., Charite, J., Hage, W.J., De Graaff, W., Deschamps, J., 1997. Hoxb-8 gain-of-function transgenic mice exhibit alterations in the peripheral nervous system. J. Neurosci. Methods 71, 11-18.

Gaunt, S.J., Coletta, P.L., Pravtcheva, D., Sharpe, P.T., 1990. Mouse *Hox3.4*: Homeobox sequence and embryonic expression patterns compared with other members of the *Hox* gene network. Development 109, 329-339.

Gilbert, S.F., 2000. Developmental biology, sixth ed. Sinauer Associates, Inc., Sunderland, Massachusetts

Gould, A., Morrison, A., Sproat, G., White, R.A.H., Krumlauf, R., 1997. Positive cross-regulation and enhancer sharing: two mechanisms for specifying overlapping *Hox* expression patterns. Genes Dev. 11, 900-913.

Goulding, M., Lanuza, G., Sapir, T., Narayan, S., 2002. The formation of sensorimotor circuits. Curr. Opin. Neurobiol. 12, 508-515.

Gowan, K., Helms, A.W., Hunsaker, T.L., Collisson, T., Ebert, P.J., Odom, R., Johnson, J.E., 2001. Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. Neuron 31, 219-232.

Gross, M.K., Dottori, M., Goulding,, M., 2002. Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. Neuron 34, 535-549.

Hallonet, M.E., Teillet, M.A., Le Douarin, N.M., 1990. A new approach to the development of the cerebellum provided by the quail-chick marker system. Development 108, 19-31.

Hao, Z., Yeung, J., Wolf, L., Doucette, R., Nazarali, A., 1999. Differential expression of Hoxa-2 protein along the dorsal-ventral axis of the developing and adult mouse spinal cord. Dev. Dyn. 216, 201-217.

Hunt, P., Krumlauf, R., 1991. Deciphering the Hox code: clues to patterning branchial regions of the head. Cell 66, 1075-1078.

Jeannotte, L., Lemieux, M., Charron, J., Poirier, F., Robertson, E.J., 1993. Specification of axial identity in the mouse: role of the *Hoxa-5 (Hox1.3)* gene. Genes Dev. 7, 2085-2096.

Jungbluth, S, Bell, E., Lumsden, A., 1999. Specification of distinct motor neuron identities by the singular activities of individual Hox genes. Development 126, 2751-2758.

Kataoka, K., Yoshitomo-Nakagawa, K., Shioda, S., Nishizawa, M., 2001. A set of Hox proteins interact with the Maf oncoprotein to inhibit its DNA binding, transactivation, and transforming activities. J. Biol. Chem. 276, 819-826.

Kessler, M., A., Yang, M., Gollomp, K., L., Jin, H., Iacovitti, L., 2003. The human tyrosine hydroxylase gene promoter. Mol. Brain Res. 112, 8–23.

Kim, C.-H., Hwang, D.-Y., Jae-Joon Park, J.-J., Kim, K.-S., 2002. A proximal promoter domain containing a homeodomain-binding core motif interacts with multiple transcription factors, including HoxA5 and Phox2 proteins, and critically regulates cell type-specific transcription of the human norepinephrine transporter gene. J. Neurosci. 22, 2579–2589.

Kinkead, R., Leblanc, M., Gulemetova, R., Lalancette-Hébert, M., Lemieux, M., Mandeville, I., Jeannotte, L., 2004. Respiratory adaptations to lung morphological defects in adult mice lacking *Hoxa5* gene function. Pediatr. Res. 56, 553-562.

Kondo, T., Zakany, J., Duboule, D., 1998. Control of colinearity in *AbdB* genes of the mouse *HoxD* complex. Molecular Cell 1, 289-300.

Krieger, K.E., Abbott, M.A., Joksimovic, M., Lueth, P.A., Sonea, I.M., Jeannotte, L., Tuggle, C.K., 2004. Transgenic mice ectopically expressing HOXA5 in the dorsal spinal cord show structural defects of the cervical spinal cord along with sensory and motor defects of the forelimb. Brain Res. Dev. Brain Res. 150, 125-139.

Krumlauf, R., 1994. *Hox* genes in vertebrate development. Cell 78, 191-201.

Larochelle, C., Tremblay, M., Bernier, D., Aubin, J., Jeannotte, L., 1999. Multiple *cis*-acting regulatory regions are required for the restricted spatio-temporal *Hox5* gene expression. Dev. Dyn. 214, 127-140.

Lee, K.J., Mendelsohn, M., Jessell, T.M., 1998. Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. Genes Dev. 12, 3394-3407.

Lee, K.J., Jessell, T.M., 1999. The specification of dorsal cell fates in the vertebrate central nervous system. Annu. Rev. Neurosci. 22, 261-294.

Liem, K.F.Jr., Tremml, G., Jessell, T.M., 1997. A role for the roof plate and its resident TGFbeta related proteins in neuronal patterning in the dorsal spinal cord. Cell 91, 127-138.

Mann, R.S., Chan, S-K., 1996. Extra specificity from extradenticle: the partnership between HOX and PBX/EXD homeodomain proteins. TIG 12, 258-262.

Mann, R.S., 1997. Why are *Hox* genes clustered? BioEssays 19, 661-664.

Marin, F., Puelles, L., 1995. Morphological fate of rhombomeres in quail/chick chimeras: a segmental analysis of hindbrain nuclei. Eur. J. Neurosci. 7, 1714-1738.

Mark, M., Lufkin, T., Vonesch, J.L., Ruberte, E., Olivo, J.C., Dolle, P., Gorry, P., Lumsden, A., Chambon, P., 1993. Two rhombomeres are altered in Hoxa-1 mutant mice. Development 119, 319-338.

Marti, E., Bumcrot, D.A., Takada, R., McMahon, A.P., 1995. Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. Nature 375, 322-325.

Matise, M., 2002. A dorsal elaboration in the spinal cord. Neuron 34, 491-493.

Meunier, D., Aubin, J., Jeannotte L., 2003. Perturbed thyroid morphology and transient hypothyroidism symptoms in *Hoxa5* mutant mice. Dev. Dyn. 227, 367-378.

Morrison, A.D., 1998. 1 + 1 = r4 and much much more. Bioessays 20, 794-797.

Murphy, S.P., Garbern, J., Odenwald, W.F., Lazzarini, R.A., Linney, E., 1988. Differential expression of the homeobox gene Hox-1.3 in F9 embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA 85, 5587-5591.

Odenwald, W.F., Taylor, C.F., Palmer-Hill, F.J., Friedrich, V.Jr., Tani, M., Lazzarini, R.A., 1987. Expression of a homeo domain protein in noncontact-inhibited cultured cells and postmitotic neurons. Genes Dev. 1, 482-496.

Oosterveen, T., Niederreither, K., Dolle, P., Chambon, P., Meijlink, F., Deschamps, J., 2003. Retinoids regulate the anterior expression boundaries of 5' Hoxb genes in posterior hindbrain. EMBO J. 22, 262-269.

Patten, I., Placzek, M., 2000. The role of Sonic hedgehog in neural tube patterning. Cell. Mol. Life Sci. 57, 1695-1708.

Pattyn, A., Goridis, C., Brunet, J-F. 2000. Specification of the central noradrenergic phenotype by the homeobox gene Phox2b. Mol. Cell Neurosci. 15, 235–243.

Pierani, A., Brenner-Morton, S., Chiang, C., Jessell, T.M., 1999. A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell 97, 903-915.

Qian, Y., Fritzsch, B., Shirasawa, S., Chen, C.L., Choi, Y., Ma, Q., 2001. Formation of brainstem (nor)adrenergic centers and first-order relay visceral sensory neurons is dependent on homeodomain protein Rnx/Tlx3. Genes Dev. 15, 2533-2545.

Rancourt, D.E., Tsuzuki, T., Capecchi, M.R., 1995. Genetic interaction between hoxb-5 and hoxb-6 is revealed by nonallelic noncomplementation. Genes Dev. 9, 108-122.

Rijli, F.M., Matyas, R., Pellegrini, M., Dierich, A., Gruss, P., Dolle, P., Chambon, P., 1995. Cryptorchidism and homeotic transformations of spinal nerves and vertebrae in Hoxa-10 mutant mice. Proc. Natl. Acad. Sci. USA 92, 8185-8189.

Sanlioglu, S., Zhang, X., Baader, S.L., Oberdick, J., 1998. Regulation of a Purkinje cell-specific promoter by homeodomain proteins: repression by engrailed-2 vs. synergistic activation by Hoxa5 and Hoxb7. J. Neurobiol. 36, 559-571.

Shanmugam, K., Green, N.C., Rambaldi, I., Saragovi, H.U., Featherstone, M.S., 1999. PBX and MEIS as non-DNA-binding partners in trimeric complexes with HOX proteins. Mol. Cell. Biol. 19, 7577-7588.

Sharpe, J., Nonchev, S., Gould, A., Whiting, J., Krumlauf, R. 1998. Selectivity, sharing and competitive interactions in the regulation of *Hoxb* genes. EMBO J. 17, 1788-1798.

Sherrard, R., M., Bower, 2002. Climbing fiber development: do neurotrophins have a part to play? The Cerebellum, 1, 265-276.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A., Krumlauf, R., 1996. Altered segmental identity and abnormal migration of motor neurons in mice lacking Hoxb-1. Nature 384, 630-634.

Studer, M., Gavalas, A., Marshall, H., Ariza-McNaughton, L., Rijli, F.M., Chambon, P., Krumlauf, R., 1998. Genetic interaction between *Hoxa1* and *Hoxb1* reveal new roles in regulation of early hindbrain patterning. Development 125, 1025-1036.

Sugihara, I., Shinoda, Y., 2004. Molecular, topographic, and functional organization of the cerebellar cortex: a study with combined aldolase C and olivocerebellar labeling. J. Neurosci. 24, 8771-8785.

Tiret, L., le Mouellic, H., Maury, M., Brulet, P., 1998. Increased apoptosis of motoneurons and altered somatotopic maps in the brachial spinal cord of Hoxc-8-deficient mice. Development 125, 279-291.

van der Hoeven, F., Zakany, J., Duboule, D., 1996. Gene transpositions in the HoxD complex reveal a hierarchy of regulatory controls. Cell 85, 1025-1035.

Wall, N.A., Jones, M., Hogan, B.L.M., Wright, C.V.E., 1992. Expression and modification of Hox2.1 protein in mouse embryos. Mech. Dev. 37, 111-120.

Xiang, M., Gan, L., Zhou, L., Klein, W.H., Nathans, J., 1996. Targeted deletion of the mouse POU domain gene Brn-3a causes selective loss of neurons in the brainstem and

trigeminal ganglion, uncoordinated limb movement, and impaired suckling. Proc. Natl. Acad. Sci. USA 93, 11950-11955.

Zakany, J., Tuggle, C.K., Patel, M., Nguyen-Huu, M.C., 1988. Spatial regulation of homeobox gene fusions in the embryonic central nervous system of transgenic mice. Neuron 1, 679-691.

CHAPTER III. Dynamic expression of murine HOXA5 protein in the central nervous system

A paper published in *Gene Expression Patterns*[1]

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Abstract

The *Hox* genes encode transcription factors that are indispensable for proper spatio-temporal patterning of the vertebrate body axes. As for other *Hox* genes, region-specific expression of *Hoxa5* appears to be important for correct function during development. In mouse, *Hoxa5* transcripts are differentially expressed in specific mesoderm-derived structures and in the most anterior domain of expression in the central nervous system (CNS), in contrast to indistinct patterns seen in the posterior CNS. However, the functional significance of any pattern of protein-coding RNAs must be verified by correlating the presence of the protein(s) and RNAs. Here we describe the dynamic pattern of HOXA5 protein during mouse embryogenesis. The HOXA5 protein is detected as early as embryonic day (E) 9.0, and is found, as development proceeds, in several mesoderm-derived structures such as prevertebrae (pv), proximal forelimb bud, scapula, lung, trachea, and gut. In addition,

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the protein shows a strikingly restricted and dynamic expression pattern in the developing CNS, and is detected in both motor neurons and interneurons between E10.5-E13.5. Moreover, this CNS region-specific HOXA5 protein pattern is more restricted than the pattern observed for the *Hoxa5* transcripts. In many mesoderm-derived tissues affected by the *Hoxa5* mutation, the expression pattern of HOXA5 protein corresponds to that of the putative functional *Hoxa5* transcript. However, in the CNS, this correlation is exclusively demonstrated in the most anterior domain of expression. Overall, the HOXA5 protein pattern is consistent with its proposed role in positional specification in mesodermal structures, as well as in the embryonic neuraxis.

1. Results and Discussion

Hox genes have important functions in regulating morphogenesis and in specifying regional identity along the embryonic axes (Krumlauf, 1994). Mutations in *Hox* genes cause homeotic transformations in the skeleton and defects in organogenesis that may impair viability. For instance, disruption of the *Hoxa5* gene causes homeotic skeletal transformations located from the third cervical (C3) to the second thoracic vertebra (T2) and alterations in the formation of the acromion (Jeannote et al., 1993; Aubin et al., 1998, 2002a). Impaired tracheal and lung morphogenesis is likely to be the cause of early postnatal lethality of *Hoxa5* mutant mice (Aubin et al., 1997). The achievement of an adult mode of digestion is delayed, and thyroid development is also affected in these mutants (Aubin et al., 1999; Meunier et al., 2003). Despite the fact that no CNS phenotype has been described for *Hoxa5* deficient animals, structural deficits of the cervical spinal cord, as well as sensory and motor

defects involving the forelimb, were reported in mice expressing a *Hoxa5* transgene in the dorsal spinal cord (Krieger et al., 2004).

Transcription of the *Hoxa5* gene produces a complex pattern of multiple mRNAs, including a short version (1.8 kb) and three larger ones (5.0 kb, 9.5 kb and 11.0 kb). *In situ* analyses using probes that detect either the three larger transcripts or all four transcripts reveals differential expression of *Hoxa5* during embryogenesis. Furthermore, structures in which sole expression of the 1.8 kb-transcript is inferred have been shown to be affected in *Hoxa5* deficient mice (Jeannotte et al., 1993; Aubin et al., 1997, 1998, 1999; Larochelle et al., 1999; Aubin et al., 2002a, 2002b). Our comparative analysis of the data reported for *Hoxa5* RNA distribution (Dony and Gruss, 1987; Gaunt et al., 1988, 1990; Aubin et al., 1998; Larochelle et al., 1999) and the localization of HOXA5 protein using a previously reported antibody (Odenwald et al., 1987; Tani et al., 1989) reveals discrepancies. The HOXA5 protein, as detected by Tani et al. (1989), appears to be present before the onset of *Hoxa5* RNA detection, and HOXA5 immunoreactivity was found in more tissues and cell types than those positive for *Hoxa5* transcripts as detected by *in situ* hybridization analysis. To define the relationship between HOXA5 protein localization and the complex *Hoxa5* transcriptional profile, we studied the expression pattern of the HOXA5 protein during embryogenesis, with a particular emphasis on the developing CNS. To do so, we produced and verified specific HOXA5 antibodies, analyzed the protein immunolocalization during embryogenesis, and found a highly dynamic expression profile of the HOXA5 protein in the developing CNS.

HOXA5-specific polyclonal antibodies were generated against a bacterially produced and purified glutathione S-transferase (GST)/mouse HOXA5 exon 1 fusion protein (see

Experimental Procedures). The *Hoxa5* exon 1 fragment used corresponded to amino acids 61-151 of the predicted HOXA5 protein (Odenwald et al., 1987). This region of HOXA5 was chosen because it is the most divergent portion of the protein among HOX proteins. For example, the HOXA5 amino acid sequence 61-151 showed 29% identity and 46% conservative substitutions with the corresponding HOXB5 sequence (http://us.expasy.org/). Immunoreactivity of the affinity-purified serum was exclusively found against the HOXA5 exon 1 moiety separated from GST (Fig. 1A; lanes 1-5; see Experimental Procedures), confirming the efficiency of purification and specificity of the antibodies. Further, these antibodies were used with protein blots to detect the presence of the HOXA5 protein in *Hoxa5*+/+ and *Hoxa5*-/- mouse embryonic tissue extracts. An immunoreactive band at about 40-42 kDa and a faint band at about 32kDa were present in the *Hoxa5*+/+ samples, but they were not seen in *Hoxa5*-/- protein extracts. Moreover, these bands were not detected by antibodies pre-absorbed with GST/HOXA5 antigen, but they were detected by GST/HOXB5- and GST-pre-absorbed antibodies (Fig. 1B and data not shown). The faint band at about 32kDa may represent fast migrating, incompletely phosphorylated protein as previously described (Odenwald et al., 1987, 1989). In addition, we performed immunohistochemical analysis of spinal cord sections from E12.5 specimens. The specificity of the detection was demonstrated by a) the complete abolition of immunoreactivity when HOXA5 antibodies were pre-absorbed with GST/HOXA5 antigen (Fig. 1D) or by omitting these antibodies during antigen detection (data not shown) and b) the retention of immunoreactivity when the antibodies were pre-absorbed with either GST/HOXB5 (Fig. 1E) or GST (data not shown) antigens or buffer (Fig. 1C). Finally, *Hoxa5*-/- embryos showed no immunoreactivity with the affinity-purified serum as assayed by immunohistochemistry (IHC)(Fig. 1F) and whole

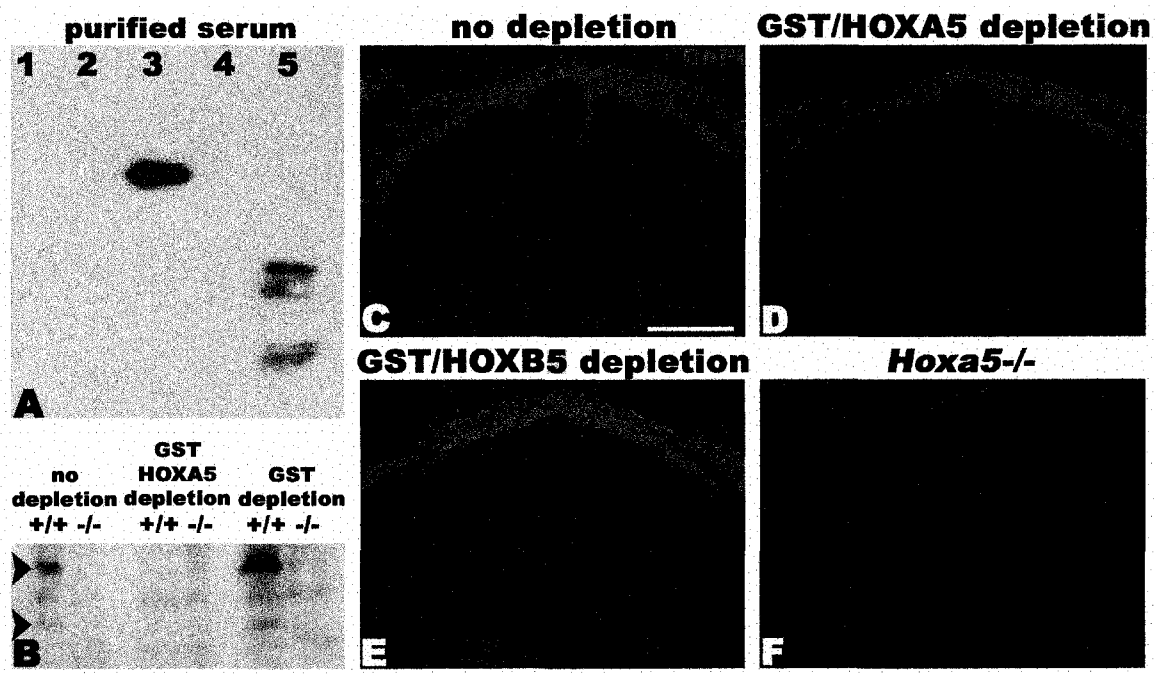


Fig. 1. Demonstration of the specificity of HOXA5 antibodies. (A) Western blot of the *E.coli* produced proteins incubated with affinity purified serum. Lanes 1-3 contain GST/HOXB5 exon I fusion protein, GST protein, and GST/HOXA5 exon I fusion protein, respectively, which were purified on a glutathione column. For lanes 4-5, purified GST/HOXA5 exon I fusion protein was re-applied to a glutathione column, and digested by thrombin to separate GST and HOXA5 exon 1 moieties. Proteins eluting from glutathione after thrombin cleavage were run in lane 5, while bound proteins (GST moiety) were then eluted with glutathione and loaded in lane 4. (B) Whole cell protein extracts from E12.5 *Hoxa5* wild-type (+/+) and *Hoxa5* homozygous mutant (-/-) embryos were resolved on SDS PAGE and stained with affinity purified serum pre-absorbed with buffer (no depletion) or indicated *E.coli* produced proteins. Upper and lower arrowheads indicate specific bands migrating approximately at 40-42 and 32 kDa, respectively. (C-F) Immunohistochemical analysis of transversely sectioned cervical spinal cord in *Hoxa5*+/+ (C-E) and *Hoxa5*-/- (F) embryos at E12.5. Affinity purified serum was preincubated with buffer (C-no depletion; F), GST/HOXA5 exon I (D), and GST/HOXB5 exon I (E) before the application on the sections. Tissue sections were stained for HOXA5 protein (red) and counterstained with DAPI (blue). Bar (C-F): 200 μm.

mount analysis (data not shown). Taken together, these data indicate that the affinity-purified antibodies are specific to HOXA5 and suitable for western blotting and IHC.

HOXA5 immunoreactivity was not detected at E7.5 (tested by IHC; data not shown), E8.5 (Fig. 2A), and E8.75 (Fig. 2B), in contrast to the reported onset of HOXA5 expression at E7.5 (Tani et al., 1989). HOXA5 expression was detected as early as E9.0 in the

developing foregut, in somites at the prospective forelimb level, and in the neural tube (Fig. 2C, C1). We observed the first detectable level of HOXA5 protein in embryos at the 14-16 somite-stage that temporally coincided with the end of turning and the closure of the anterior neuropore (see Experimental Procedures for embryo staging). Expression was maintained in the same structures at E9.5 (Fig. 2D, D1). HOXA5 immunoreactivity was localized in cells of the developing lung, in the proximity of the laryngo-tracheal groove, and in the ventral neural tube (Fig. 2D2, D3). Our analysis of initiation of HOXA5 protein expression reveals that, in the structures analyzed, the protein is expressed in the structures where *Hoxa5* RNAs were detected (Dony and Gruss, 1987; Larochelle et al., 1999) but with a delay of one-half to one embryonic day.

HOXA5 expression was maintained and refined as development proceeded from E9.5 onward. At E10.5, HOXA5 was detected in the developing foregut and spinal cord. Expression in somites was confined to a specific rostral-caudal region, approximately between somite 6-7 and somite 13-14, offset caudally compared to expression in the neural tube. HOXA5 was also detected in the developing scapula and lung (Fig. 2E, E1, E2). At E11.5, HOXA5 immunoreactivity was retained in these structures with a new expression domain in the caudal hindbrain. Moreover, HOXA5-positive cells were found in lung and tracheal mesenchyme but not in epithelium (data not shown). In addition, the gut and proximal forelimb bud were strongly immunoreactive (Fig. 2F, F1-F3). The analysis of HOXA5 expression pattern at E10.5 and E11.5 reveals several important features. First, the protein closely follows the deduced expression pattern of the 1.8 kb transcript in the mesoderm-derived structures affected by the *Hoxa5* mutation, confirming and extending the

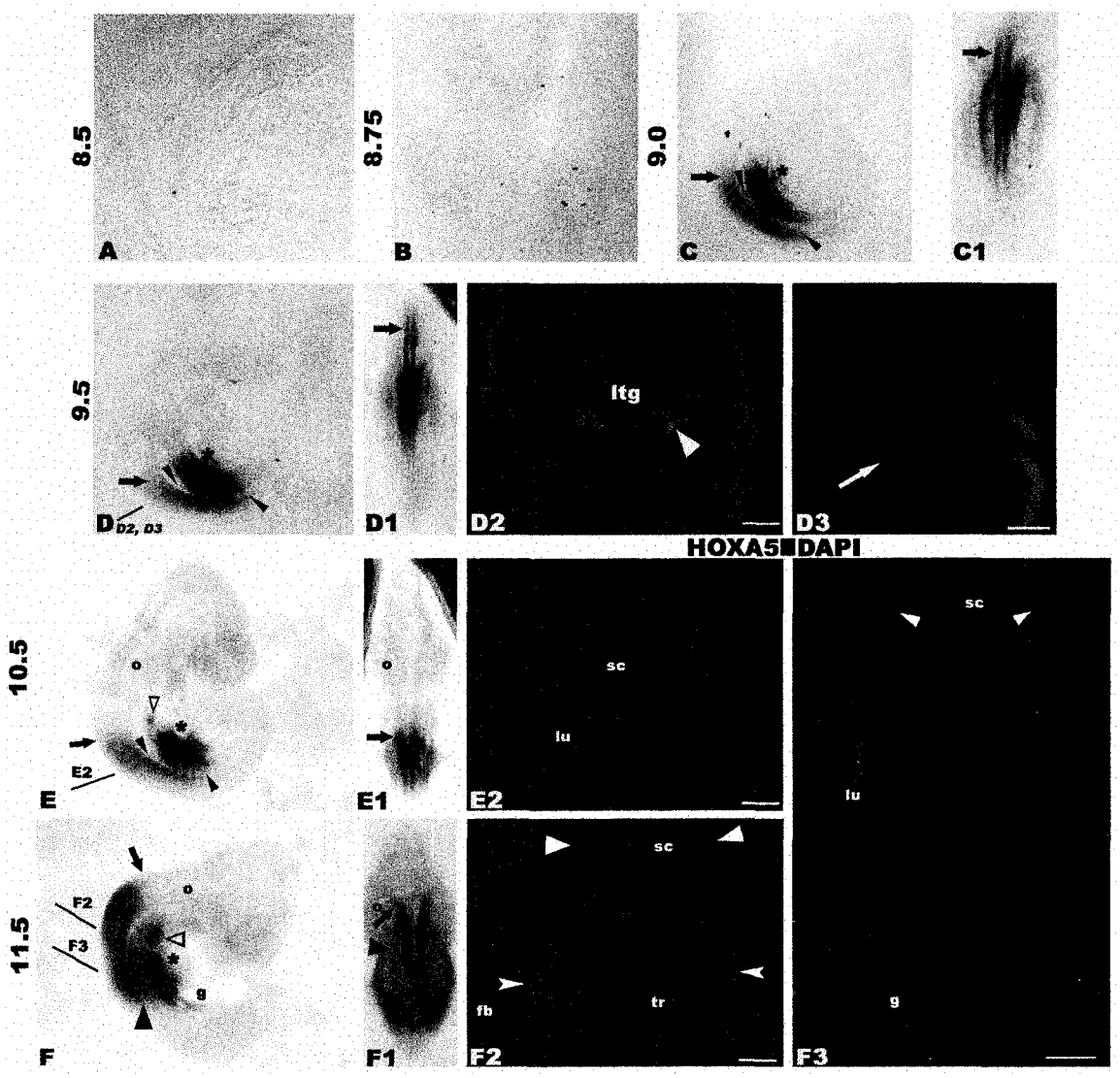
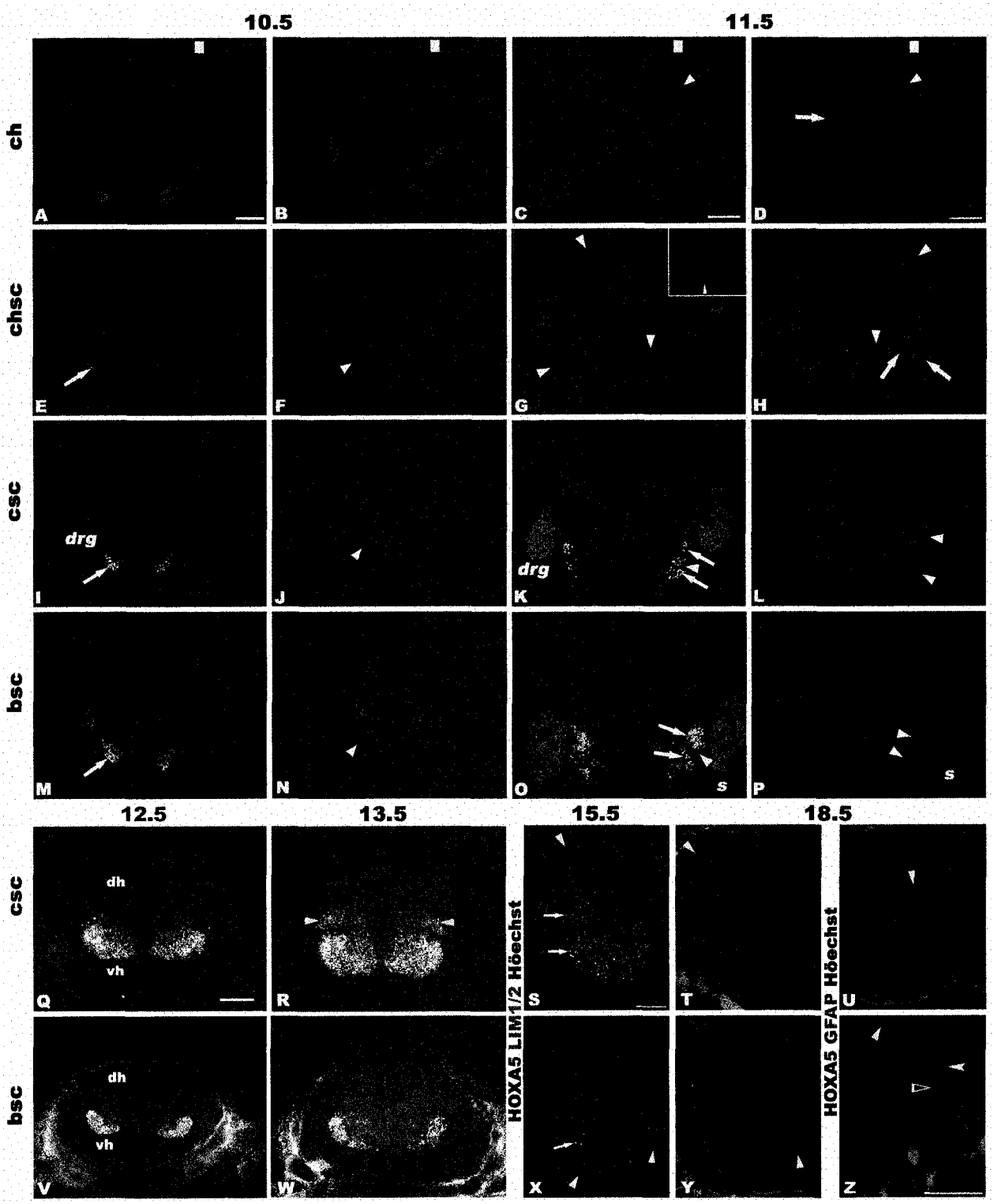


Fig. 2. Early embryonic expression profile of HOXA5. The expression was assayed in *Hoxa5*+/+ embryos by whole mount IHC at E8.5 (A), E8.75 (B), E9.0 (C, C1), E9.5 (D, D1), E10.5 (E, E1), and E11.5 (F, F1) and IHC on transverse sections stained for HOXA5 protein (red) and counterstained with DAPI (blue) at E9.5 (D2, D3), E10.5 (E2), and E11.5 (F2, F3). The approximate levels of sections in D2-D3, E2, and F2-F3 are shown by the black lines in D, E, and F, respectively. C, C1, D, D1-D3: Asterisks and arrows indicate HOXA5 expression in developing foregut and neural tube, respectively. Arrowheads depict the expression in somites (black) and developing lung bud (white). E, E1, E2, F, F1-F3: Asterisk and arrows indicate HOXA5 expression in the developing foregut and the anterior expression boundary in the neural tube, respectively. Black arrowheads show the anterior and posterior expression boundaries in somites. An open arrowhead depicts the protein localization in the developing scapula. White arrowheads and chevrons indicate HOXA5 immunoreactivity in the somites and proximal forelimb bud, respectively. g, gut; fb, forelimb bud; ltg, laryngo-tracheal groove; lu, lung; o, otic vesicle; sc, spinal cord; tr, trachea. Bars (E2, F2 and F3): 200 μm; (D2, D3): 50 μm.

functional significance of this transcript in the development of these systems (Larochelle et al., 1999). Second, *Hoxa5-/-* embryos do not express any form of HOXA5 protein detectable with our antibodies (Fig. 1F and data not shown) confirming the antibody specificity indicated by pre-absorption studies. Third, HOXA5 undergoes a neuronal expansion into caudal hindbrain between E10.5 and E11.5, reminiscent of the retinoic acid-dependent neuronal expansion of *5' Hoxb* genes (Oosterveen et al., 2003, 2004).

We next compared neuronal HOXA5 expression pattern at E10.5 and E11.5 on CNS transverse sections and defined which cell types expressed HOXA5. ISL1/2 was used as a generic marker for motor neurons and spinal cord dorsal interneuron (dI) 3 populations, whereas LIM1/2 was used as a marker for several classes of interneurons (Pierani et al., 1999; Jessell, 2000; Pierani et al., 2001; Gross et al., 2002; Müller et al., 2002; Helms and Johnson, 2003). HOXA5 expression was not detected in caudal hindbrain at E10.5 (Fig. 3A, B) whereas expression was observed in dorsal caudal hindbrain at E11.5. At this stage and position, HOXA5 was outside of ISL1/2 expression domain (Fig. 3C) but was detected in a subset of LIM1/2-positive cells (Fig. 3D). At E10.5, a few HOXA5-positive cells were detected within a domain of ISL1/2 expression in the ventral neural tube at the junction of caudal hindbrain and the spinal cord (Fig. 3E), where there was no substantial expression of HOXA5 within LIM1/2-positive cells (Fig. 3F). In contrast, at E11.5, HOXA5 was not detected within a domain of ISL1/2 expression (Fig. 3G), while there was HOXA5 expression within LIM1/2-positive cells at the junction of caudal hindbrain and the spinal cord (Fig. 3H). In addition, at this axial level, after 2 hours of BrdU labeling, HOXA5 expression was detected outside but in close proximity to dividing (BrdU-positive) neural progenitor cells (Fig. 3G-inset). In ventral cervical spinal cord at E10.5, HOXA5 was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exclusively expressed in motor neurons (ISL1/2-positive cells; Fig. 3I) and largely detected outside of LIM1/2-positive cells (Fig. 3J). However, in ventral cervical spinal cord at E11.5, HOXA5 was not exclusively expressed in motor neurons (ISL1/2-positive cells; Fig. 3K) but still remained outside of the LIM1/2 expression domain (Fig. 3L). A similar expression pattern was observed in the brachial spinal cord at E10.5 and E11.5 (Fig. 3M-P). Thus, from E10.5 to E11.5, HOXA5 expression is dynamic, and found in interneurons at caudal hindbrain levels and motor neurons in the spinal cord as well as in other cell types in the developing neural tube.

The HOXA5 protein pattern was also dynamic during late gestation, showing a distinct expression profile in the cervical and brachial spinal cord. HOXA5 was expressed in the ventral horn only at both spinal cord levels at E12.5 (Fig. 3Q, V). A new expression domain was formed dorsomedially and dorsolaterally at the cervical level, while HOXA5 expression was maintained in the ventral horn at both spinal cord levels at E13.5 (Fig. 3R, W). This dorsomedial and dorsolateral expression pattern was also observed at E15.5 and E18.5 (Fig. 3S, T, U). Starting at E15.5, new HOXA5-positive cells were detected in the most dorsal region of the spinal cord at the cervical (but not brachial) level, and in a medial position within the ventral horn at the brachial level (Fig. 3S, X). Moreover, HOXA5 expression was found in a subset of LIM1/2-positive cells in the ventral horn and the cells immediately dorsal to the ventral horn at the cervical level (Fig. 3S). However, at the brachial level, HOXA5 immunoreactivity was detected mainly outside of LIM1/2-positive cells (Fig. 3X). At E18.5, similar expression of HOXA5 was seen at both spinal cord levels while we observed few if any LIM1/2-positive cells (Fig. 3T, Y). ISL1/2 was detected neither at E15.5 nor E18.5 (data not shown). No HOXA5 immunoreactivity was seen in astrocytes, although

we occasionally detected HOXA5-positive cells in vicinity of GFAP-expressing cells at both spinal cord levels at E18.5 (Fig. 3U, Z and data not shown). In addition, we did not detect HOXA5 in dorsal root ganglia (DRG) at any stage examined (Fig. 2, 3, 5).

We wished to precisely determine the anterior-posterior domain of HOXA5 expression and the cell types expressing the protein in the developing CNS at E13.5, the stage at which many neuronal cells have been specified and are settled into definitive positions (Varela-Echavarría et al., 1996; Sockanathan and Jessell, 1998; Jessell, 2000). HOXA5 was expressed from the caudal hindbrain to approximately the level of pv6 along the anterior-posterior axis (Fig. 4). HOXA5 immunoreactivity was initially detected dorsally within the most anterior expression domain, changing to medial and ventral toward the posterior expression boundary. Further, we detected a highly dynamic dorso-ventral expression profile of HOXA5 with a detailed examination along the anterior-posterior neuraxis. The protein was detected in caudal hindbrain changing from dorsal to ventral as hindbrain

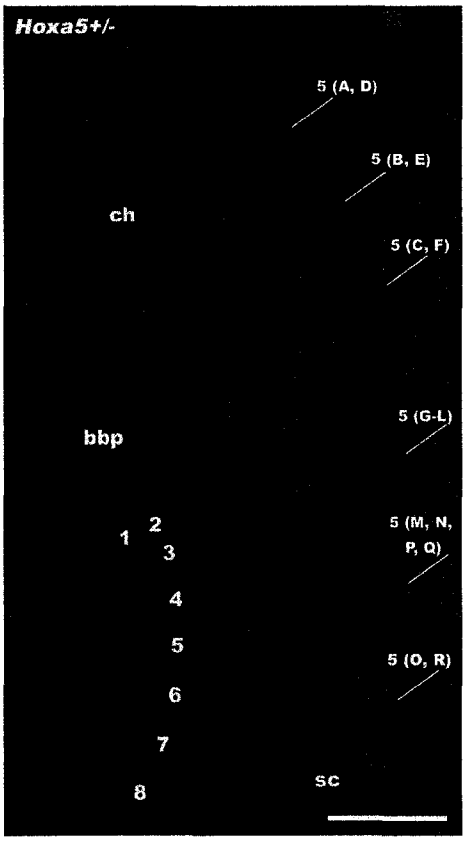
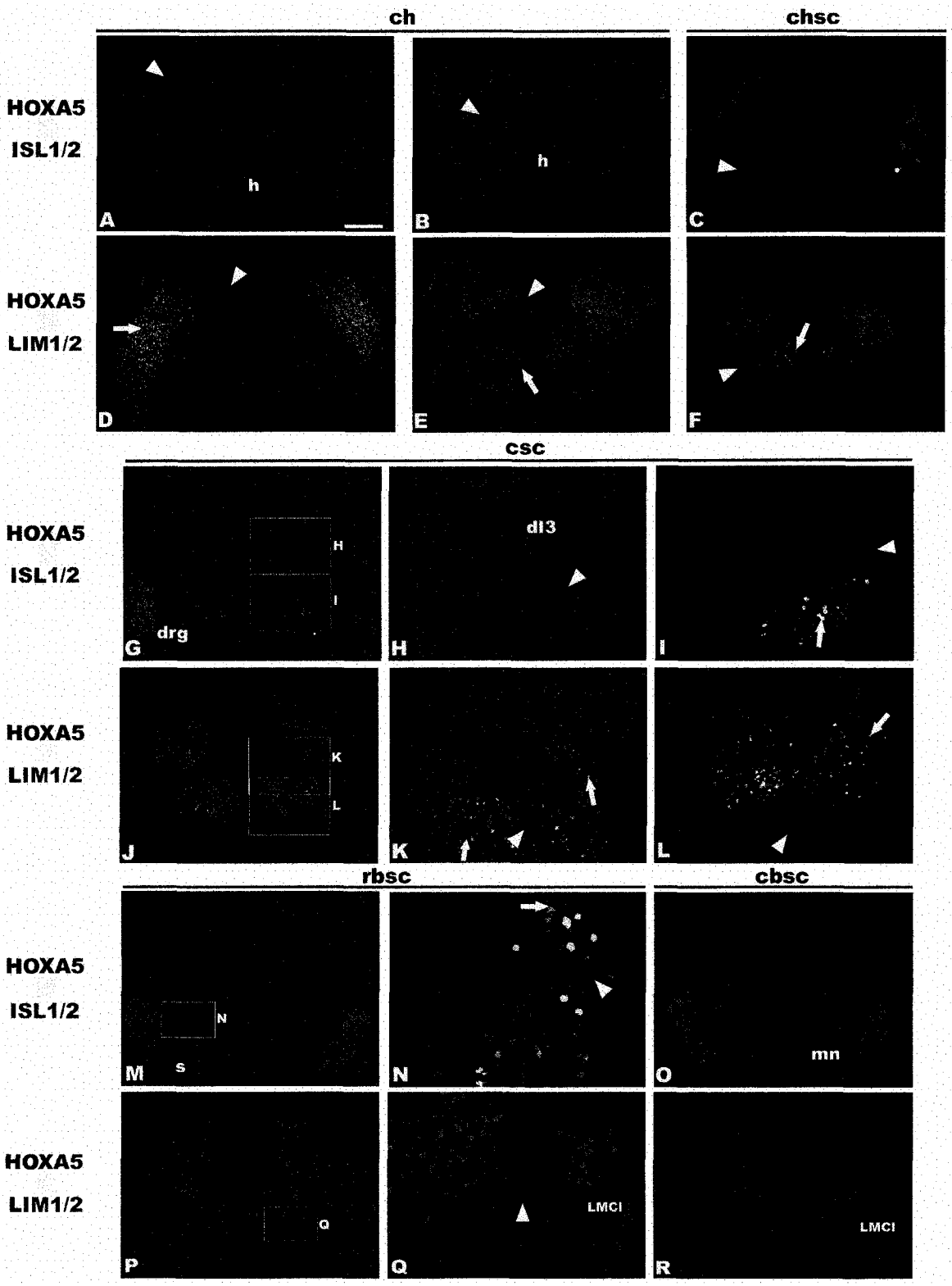


Fig. 4. Dynamic expression of HOXA5 in developing CNS at E13.5. A midsagittal section of a *Hoxa5*+/- embryo was labeled for HOXA5 protein (red) and counterstained with DAPI (blue). A similar pattern of expression was seen for *Hoxa5*+/+ embryos, but the specimen shown here contained the most complete, and therefore representative, pattern of expression. White lines show an approximate level of sections in Fig. 5. 1-8, prevertebrae 1-8, respectively; bbp, basiooccipital bone primordium; ch, caudal hindbrain; sc, spinal cord. Bar: 500 μm.

merged into the spinal cord. The anterior expression boundary of HOXA5 coincided with the anterior-posterior domain of the hypoglossal nucleus in cell types other than motor neurons (ISL1/2-positive cells)(Fig. 5, A-C). At the same hindbrain level, the protein was detected in a dorso-lateral subset of LIM1/2-positive interneurons as well as in other cell types (Fig. 5D-F). In the cervical spinal cord, HOXA5 was not exclusively expressed in motor neurons (ISL1/2-positive cells), and was largely expressed outside of dI3 interneurons (ISL1/2-positive cells)(Fig. 5G-I). Protein expression was detected in a subset of LIM1/2-positive interneurons and in a subset of LIM1/2-positive cells immediately dorsal to the ventral horn, as well as in other cell types (Fig. 5J-L). In rostral brachial spinal cord, HOXA5 was not exclusively expressed in motor neurons (ISL1/2-positive cells)(Fig. 5M, N). At the same spinal cord level, HOXA5 was detected neither in cells of lateral motor column lateral (LMC_l) (ventro-lateral LIM1-positive cells) nor interneurons (ventro-medial LIM1/2-positive cells)(Fig. 5P, Q). The expression of HOXA5 was not detected in caudal brachial spinal cord (Fig. 5O, R). HOXA5 localization in rostral but not caudal brachial spinal cord is consistent with the expression pattern of chick HOXC5 (Dasen et al., 2003). Thus, HOXA5 shows a region-specific expression pattern spanning the area from the anterior-posterior domain of the hypoglossal nucleus in caudal hindbrain to rostral brachial spinal cord. Moreover, the protein is expressed in interneurons but not motor neurons in caudal hindbrain, while in rostral brachial spinal cord HOXA5 is expressed in some motor neurons but not in the LMC_l.

In the CNS, the protein expression pattern is more restricted than the *Hoxa5* RNA pattern reported by several groups (Dony and Gruss, 1987; Gaunt et al., 1990; Aubin et al., 1998; Larochelle et al., 1999). Using probes recognizing all *Hoxa5* transcripts, RNA expression was detected in the posterior spinal cord with the anterior boundary at pv7 level at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E9.0 (Dony and Gruss, 1987) and in the posterior spinal cord region as well as the anterior neural tube at E12.5 (Gaunt et al., 1990; Larochelle et al., 1999). More recently, the posterior spinal cord pattern was refined using a probe that exclusively recognized the larger transcripts. These transcripts were detected in the spinal cord as well as in a more anterior domain of the neural tube (Aubin et al., 1998; Larochelle et al., 1999). Due to the broad expression of the larger transcripts, a specific CNS expression pattern of the 1.8 kb transcript cannot be deduced as in mesoderm-derived structures, except in the most anterior domain of expression in the neural tube at E10.5 and E12.5 where larger transcript-specific probes do not show a hybridization signal (Larochelle et al., 1999). Therefore, in the developing CNS, there is no sharply defined connection between the deduced expression pattern of the 1.8 kb transcript and the protein localization, which could suggest a specific spatial domain of HOXA5 expression affected in *Hoxa5* mutant embryos. Transcript and protein patterns may or may not correlate in the CNS, as post-transcriptional and translational mechanisms have been reported for control of *Hox* expression (Brend et al., 2003). However, others have reported HOXA5 protein expression in structures for which we do not detect protein, such as developing forebrain as well as in the posterior CNS and peripheral nervous system (Tani et al., 1989). Further, Tani and colleagues did not detect a region-specific expression pattern – a hallmark of *Hox* gene expression. The antibodies reported here clearly have high specificity along with single-cell level sensitivity and reveal that: a) the HOXA5 protein has an anterior boundary of expression similar to that of *Hoxa5* transcripts in both the developing prevertebrae and in the CNS (Dony and Gruss, 1987; Gaunt et al., 1988, 1990; Aubin et al., 1998; Larochelle et al., 1999); b) the HOXA5 expression pattern correlates with the specific pattern deduced for the 1.8 kb transcript in mesodermal structures shown to be affected by

the *Hoxa5* mutation (Jeannotte et al., 1993; Aubin et al., 1997, 1998; Larochelle et al., 1999; Aubin et al., 2002a); c) the expression of HOXA5 and larger transcripts are not correlated, as the posterior limit of HOXA5 CNS expression is in the caudal brachial spinal cord, while the larger *Hoxa5* transcripts are present throughout the spinal cord. Therefore, we believe that the protein pattern reported here is strong evidence for a region-specific *in vivo* distribution of murine HOXA5 protein in both CNS and some mesodermal tissues. Taken together, it is tempting to speculate that HOXA5 may play an important role in specifying regional identity along the embryonic neuraxis as it does for mesoderm-derived structures (Jeannotte et al., 1993; Aubin et al., 1998).

2. Experimental Procedures

2.1. Animal handling procedures

Animal handling procedures were conducted under the approval of the Iowa State University Animal Care and Use Committee, and conform to NIH recommended guidelines. Animals were sacrificed by CO_2 asphyxiation.

2.2. Antigen production, immunization, and antibody purification

GST/HOXA5 exon 1 and GST/HOXB5 exon I fusion proteins and GST protein were expressed in *E. coli* $DH_5\alpha$ cells. Proteins were affinity purified by incubation with Glutathione-Sepharose 4B, and eluted with glutathione according to manufacturer's instructions (Amersham). Immunization of two rabbits with GST/HOXA5 exon I fusion protein was carried out by the Cell and Hybridoma facility at Iowa State University. All bleeds from both rabbits (7 and 11 bleeds) showed specific immunoreactivity against

HOXA5 exon I moieties in western blot screening (data not shown). Two bleeds that showed the most intense signal were then used for further work. The crude sera were purified by three consecutive depletions using the following proteins/extracts coupled to CNBr-preactivated Sepharose (Sigma) according to the manufacturer's instruction. The sera were passed over columns that had covalently bound a) whole bacterial extract containing GST/HOXB5 exon I fusion protein, b) purified GST/HOXB5 exon I fusion protein or c) purified GST protein, followed by affinity purification on a column with covalently bound purified GST/HOXA5 exon I fusion protein. In addition, the GST/HOXA5 exon I fusion protein bound to glutathione beads was digested by thrombin protease following manufacturer's instructions (Amersham).

2.3. Embryo staging, Western blot, whole mount, and immunohistochemistry

Embryos were staged using different morphological characteristics according to Kaufman (1992) and EMAP Edinburgh Mouse Atlas Project (http://genex.hgu.mrc.ac.uk/Atlas/intro.html). Western blot and whole mount preparations were essentially done as previously described (Harlow and Lane, 1988; Hogan et al., 1994). For immunohistochemical analysis, embryos were dissected away from placenta and yolk sac in a cold phosphate buffered saline and fixed in 4% paraformaldehyde from 30 minutes to overnight depending upon the embryonic size, cryoprotected in 30% sucrose overnight and sectioned at 20μm. Tissue sections were incubated with a blocking solution containing 5% Normal Goat Serum (Sigma), 1% BSA, and 0.4% TritonX-100 for a minimum of 60 minutes and covered with rabbit anti-HOXA5 primary antibodies overnight at 4^0C. A goat anti-rabbit Alexa Fluor 594 (Molecular Probes) was used as a secondary antibody. Tissue sections were

counterstained with either 4', 6-Diamidino-2-phenylindole (DAPI; Molecular Probes) or 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloride hydrate (Höechst 33258; Sigma) and mounted with VectaShield Mounting Medium (Vector Laboratories). Antibodies included: mouse anti-ISL1/2, mouse anti-LIM1/2, mouse anti-5-bromo-2'-deoxyuridine (BrdU) (Developmental Studies Hybridoma Bank), and mouse anti-Glial Fibrillary Acidic Protein (GFAP; Sigma). For double-labeled immunofluorescence, tissue sections were incubated in the blocking solution described above. Either ISL1/2 or LIM1/2 were detected by a goat anti-mouse Alexa Fluor 488 secondary antibodies (Molecular Probes) at E10.5. M.O.M. kit (Vector Laboratories) was used for double-labeled immunofluorescence experiments at E11.5, E13.5, E15.5, and E18.5 according to manufacturer instructions. Sections were observed and photographed using a Zeiss Axioplan II fluorescent microscope equipped with Axiocam-digital camera. Images were processed in Adobe Photoshop. In all cases, representative sections and samples are shown; described patterns were seen in at least three independent specimens.

2.4. BrdU labeling and detection

This was essentially done as previously described (Moran-Rivard et al., 2001), except that HOXA5 antibody was used at 1:200, and then BrdU was detected by using a 0.67 N HCl pre-treatment.

Acknowledgments

We thank Dr. Brigid Hogan for providing the *Gst/Hoxb5* exon I construct (Wall et al., 1992), and acknowledge Dr. Matt Abbott for assistance in establishment of

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References

Aubin, J., Lemieux, M., Tremblay, M., Bérard, J., Jeannotte, L., 1997. Early postnatal lethality in *Hoxa-5* mutant mice is attributable to respiratory tract defects. Dev. Biol. 192, 432-445.

Aubin, J., Lemieux, M., Tremblay, M., Behringer, R.R., Jeannotte, L., 1998. Transcriptional interferences at the *Hoxa4/Hoxa5* locus: Importance of correct *Hoxa5* expression for the proper specification of the axial skeleton. Dev. Dyn. 212, 141-156.

Aubin, J., Chailler, P., Ménard, D., Jeannotte, L., 1999. Loss of *Hoxa5* gene function in mice perturbs intestinal maturation. Am. J. Physiol. 277, C965–C973.

Aubin, J., Lemieux, M., Moreau, J., Lapointe, J., Jeannotte, L., 2002a. Cooperation of *Hoxa5* and *Pax1* genes during formation of the pectoral girdle. Dev. Biol. 244, 96-113.

Aubin, J., Déry, U., Lemieux, M., Chailler, P., Jeannotte L., 2002b. Stomach regional specification requires *Hoxa5*-driven mesenchymal-epithelial signaling. Development 129, 4075-4087.

Brend, T., Gilthorpe, J., Summerbell, D., Rigby, P.W., 2003. Multiple levels of transcriptional and post-transcriptional regulation are required to define the domain of *Hoxb4* expression. Development 130, 2717-2728.

Dasen, J.S., Liu, J-P., Jessell, T.M., 2003. Motor neuron columnar fate imposed by sequential phases of Hox-c activity. Nature 425, 926-933.

Dony, C., Gruss, P., 1987. Specific expression of the *Hox 1.3* homeo box gene in murine embryonic structures originating from or induced by the mesoderm. EMBO J. 6, 2965-2975.

Gaunt, S.J., Sharpe, P.T., Duboule, D., 1988. Spatially restricted domains of homeo-gene transcripts in mouse embryos: relation to a segmented body plan. Development 104, 169-179.

Gaunt, S.J., Coletta, P.L., Pravtcheva, D., Sharpe, P.T., 1990. Mouse *Hox3.4*: Homeobox sequence and embryonic expression patterns compared with other members of the *Hox* gene network. Development 109, 329-339.

Gross, M., Dottori, M., Goulding, M., 2002. *Lbx1* specifies somatosensory association interneurons in the dorsal spinal cord. Neuron 34, 535-549.

Harlow, E., Lane, D., 1988. Antibodies: A laboratory manual, first ed. Cold Spring Harbor Laboratory Press, New York.

Helms, A., Johnson, J., 2003. Specification of dorsal spinal cord interneurons. Curr. Opin. Neurobiol.13, 42-49.

Hogan, B., Constantini, F., Beddington, R., Lacy, E., 1994. Manipulating the mouse embryo: A laboratory manual, second ed. Cold Spring Harbor Laboratory Press, New York.

Jeannotte, L., Lemieux, M., Charron, J., Poirier, F., Robertson, E.J., 1993. Specification of axial identity in the mouse: role of the *Hoxa-5 (Hox1.3)* gene. Genes Dev. 7, 2085-2096.

Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev. Genet. 1, 20-29.

Kaufman, M.H., 1992. The atlas of mouse development, first ed. Academic Press, San Diego.

Krieger, K.E., Abbott, M.A., Joksimovic, M., Lueth, P.A., Sonea, I.M., Jeannotte, L., Tuggle, C.K., 2004. Transgenic mice ectopically expressing HOXA5 in the dorsal spinal cord show structural defects of the cervical spinal cord along with sensory and motor defects of the forelimb. Brain Res. Dev. Brain Res. 150, 125-139.

Krumlauf, R., 1994. *Hox* genes in vertebrate development. Cell 78, 191-201.

Larochelle, C., Tremblay, M., Bernier, D., Aubin, J., Jeannotte, L., 1999. Multiple *cis*-acting regulatory regions are required for the restricted spatio-temporal *Hoxa5* gene expression. Dev. Dyn. 214, 127-140.

Meunier, D., Aubin, J., Jeannotte L., 2003. Perturbed thyroid morphology and transient hypothyroidism symptoms in *Hoxa5* mutant mice. Dev. Dyn. 227, 367-378.

Moran-Rivard, L., Kagawa, T., Saueressig, H., Gross, M.K., Burrill, J., Goulding, M., 2001. *Evx1* is a postmitotic determinant of V0 interneuron identity in the spinal cord. Neuron 29, 385–399.

Müller, T., Brohmann, H., Pierani, A., Heppenstall, P.A., Lewin, G.R., Jessell, T.M., Birchmeier, C., 2002. The homeodomain factor Lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron 34, 551-562.

Odenwald, W.F., Taylor, C.F., Palmer-Hill, F.J., Friedrich, V.Jr., Tani, M., Lazzarini, R.A., 1987. Expression of a homeo domain protein in noncontact-inhibited cultured cells and postmitotic neurons. Genes Dev. 1, 482-496.

Odenwald, W.F., Garbern, J., Arnheiter, H., Tournier-Lasserve, E., Lazzarini, R.A., 1989. The *Hox1.3* homeo box protein is a sequence-specific DNA-binding phosphoprotein. Genes Dev. 3, 158-172.

Oosterveen, T., Niederreither, K., Dolle, P., Chambon, P., Meijlink, F., Deschamps, J., 2003. Retinoids regulate the anterior expression boundaries of 5' *Hoxb* genes in posterior hindbrain. EMBO J. 22, 262-269.

Oosterveen, T., Meijlink, F., Deschamps, J., 2004. Expression of Retinaldehyde dehydrogenase II and sequential activation of *5' Hoxb* genes in the mouse caudal hindbrain. Gene Expr. Patterns 4, 243-247.

Pierani, A., Brenner-Morton, S., Chiang, C., Jessell, T.M., 1999. A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell 97, 903-115.

Pierani, A., Moran-Rivard, L., Sunshine, M.J., Littman, D.R., Goulding, M., Jessell, T.M., 2001. Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. Neuron 29, 367-384.

Sockanathan, S., Jessell, T.M., 1998. Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. Cell 94, 503-514.

Tani, M., Odenwald, W.F., Lazzarini, R.A., Friedrich, V.L.Jr., 1989. Progressive restriction in the distribution of the *Hox-1.3* homeodomain protein during embryogenesis. J. Neurosci. Res. 24, 457-469.

Varela-Echavarría, A., Pfaff, S.L., Guthrie, S., 1996. Differential expression of LIM homeobox genes among motor neuron subpopulations in the developing chick brain stem. Mol. Cell. Neurosci. 8, 242-257.

Wall, N.A., Jones, M., Hogan, B.L.M., Wright, C.V.E., 1992. Expression and modification of Hox 2.1 protein in mouse embryos. Mech. Dev. 37, 111-120.

CHAPTER IV. Role of murine HOXA5 in development of the inferior olivary nucleus

A paper to be submitted to *Developmental Biology*

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Abstract

Inferior olivary neurons generated at the rhombic lip undertake a lengthy migration to settle in the ipsilateral ventro-caudal hindbrain and send their axons, referred to as climbing fibers, to the contralateral cerebellum. Here we show that HOXA5 expression is confined to the caudal region of the developing inferior olivary nucleus (ION) and dorsal lamella of the dorsal accessory olive (DAO) subnucleus in fully laminated ION. Furthermore, the ION can be transiently defined by a combinatorial expression of BRN3A and LIM1/2 transcription factors that may belong to a new dorsal neuron cell type in the caudal hindbrain defined in this study. Although HOXA5 is dispensable for the transcriptional code of ION up to embryonic day (E)16.5, the protein expression is crucial to preserve BRN3A expression in the dorsal lamella of DAO at E18.5. To date, this is the first report for expression of any *Hox5* paralog in ION. Our results suggest that HOXA5 plays a strong role in maintaining the normal transcriptional code for ION, which may affect establishment of connectivity, maturation, and synaptic stabilization of climbing fibers developed postnatally.

Introduction

Hox genes are prime candidates for providing anterior-posterior (AP)-positional information to cells at a given axial level. Mutations in *Hox* genes lead to homeotic transformations of body segments along the AP axis and alterations in organogenesis during development. Several studies showed a crucial role of *Hox* genes for the proper development of the central nervous system (CNS). In the spinal cord, for instance, *Hoxc8* and *Hoxd10* are essential for the normal development of motoneurons that control forelimb and hindlimb movements, respectively (Carpenter et al., 1997; Tiret et al.,1998). In the hindbrain, *Hoxb1* and *Hox3* genes are required and sufficient for the specification of branchial and somatic motoneurons in rhombomere (r) 4 and r5, respectively (Goddard et al., 1996; Studer et al., 1996; Bell et al., 1999; Gaufo et al., 2000; Gaufo et al., 2003; Guidato et al., 2003). In addition, particular combinations of *Hox1-3* paralog genes in r4 and 5 are crucial for the generation of precursors of visceral sensory interneurons (Gaufo et al., 2004). These instances of motor and interneuron specifications exemplify a phenomenon of spatial colinearity whereby a chromosomal location of *Hox* genes is correlated to their expression and function along the AP axis (Krumlauf, 1994). Although *Hox5* genes are expressed in the caudal hindbrain (Dony and Gruss, 1987; Gaunt et al., 1988, 1990; Oosterveen et al., 2003; Joksimovic et al., 2005), a role of any *Hox5* paralog in development of this structure has not been described.

The inferior olivary nucleus (ION) is a caudal hindbrain structure made of three subdivisions: the medial accessory olive (MAO), dorsal accessory olive (DAO), and principal olive (PO). The DAO includes the dorsal and ventral lamellae that are connected laterally forming a twisted V-shaped structure (Azzizi et al., 1987). The ION is the sole

source of climbing fibers that project to the contralateral cerebellum. This climbing fiber input is critical for cerebellar function including motor learning and control (Sherrard and Bower, 2002). Several studies reported developmental profiles of ION during embryogenesis. In the dorsal hindbrain, numerous neuronal populations are generated, at the level of the upper and lower rhombic lip. The lower (caudal) lip undergoes a series of morphological transformations to form the highly proliferative precerebellar neuroepithelium (pn). Neurons generated by the pn migrate out sequentially, along divergent pathways, to form five precerebellar nuclei: the ION, the external cuneate and lateral reticular nuclei in the medulla, and the pontine and reticulotegmental nuclei in the pons (Altman and Bayer, 1987). In mice, the ION population is generated at E10-11 in the rhombic lip of the pseudorhombomeres "r7-11" (the last rhombomere 7; Bloch-Gallego et al., 1999; Cambronero and Puelles, 2000). Inferior olive neurons then undergo a lengthy migration through a deep parenchymal pathway and stop migrating ipsilaterally, aggregating near the floor plate at E13, while their axons cross the floor plate and start projecting towards the contralateral hemicerebellum Altman and Bayer, 1987; Bloch-Gallego et al., 1999; Cambronero and Puelles, 2000). We have previously shown a dynamic and restricted expression of the HOXA5 protein along the AP and dorso-ventral (DV) axis of developing caudal hindbrain between E11.5-13.5 (Joksimovic et al., 2005). Therefore, HOXA5 expression temporally correlates to that of ION development.

In this study, we examined the role of *Hoxa5* gene in development of the ION through analysis of the expression of dorsal interneuron markers in the caudal hindbrain (Gross et al., 2002; Müller et al., 2002; Helms and Johnson, 2003). Firstly, we define a new dorsal neuron cell type in the hindbrain relative to those described for the spinal cord.

Secondly, our study reveals a temporal window within which a transcriptional code for inferior olive neurons that coexpress BRN3A and LIM1/2. Thirdly, analysis of a *Hoxa5* loss-of-function mutation in embryonic mice showed that this gene is required to maintain BRN3A expression in the dorsal lamella of DAO at E18.5. However, *Hoxa5* is dispensable for this ION transcriptional code up to E16.5. Taken together, these findings reveal that *Hoxa5* has a role in maintaining normal transcriptional code of the ION and contribute to better understanding of the role of *Hox5* genes in specification of the caudal hindbrain structures.

Materials and Methods

Animal handling procedures

Animal handling procedures were conducted under the approval of the Iowa State University Animal Care and Use Committee, and conform to NIH recommended guidelines. Animals were sacrificed by CO_2 asphyxiation.

Immunohistochemistry

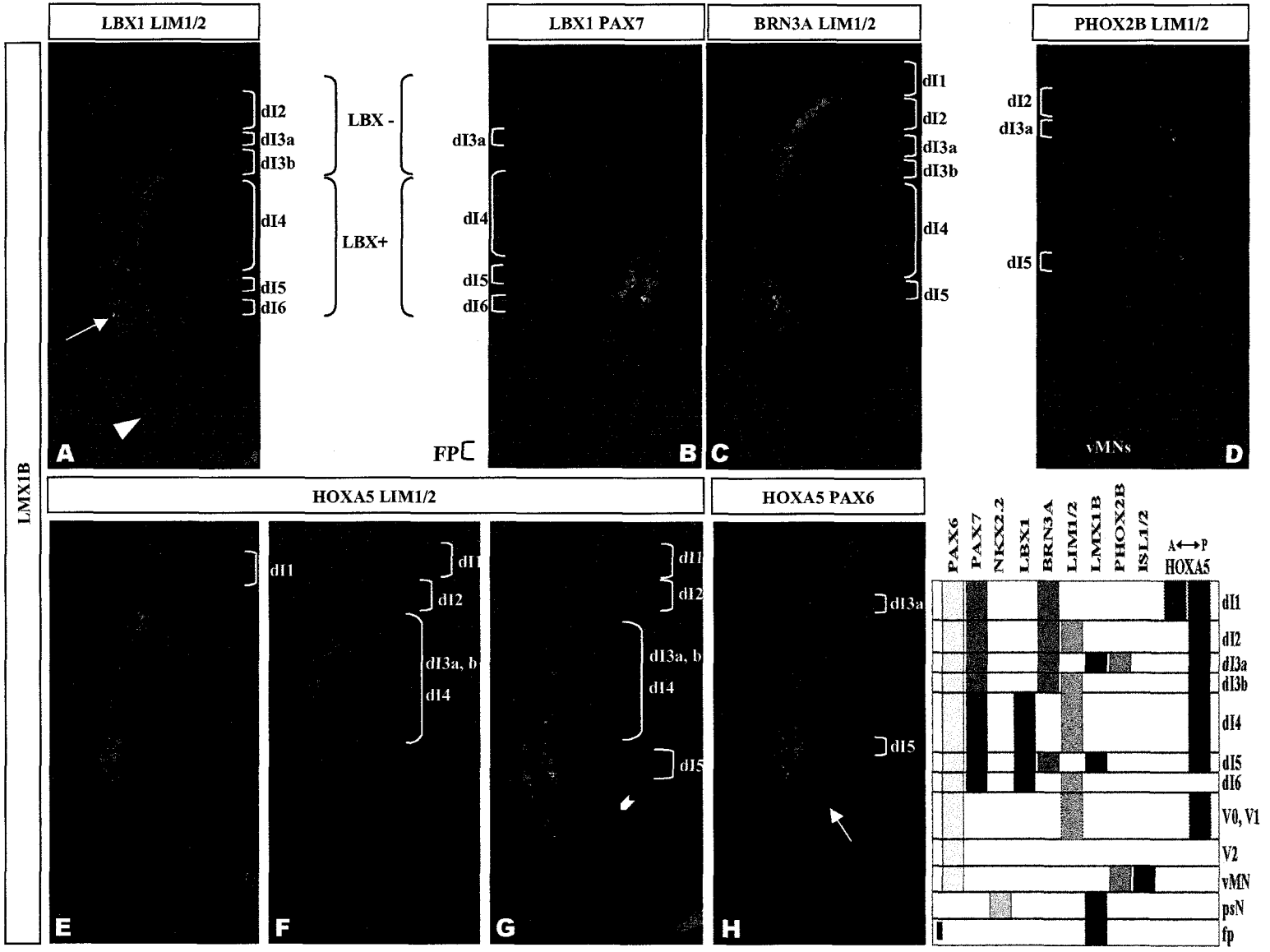
Twenty-micrometer adjacent cryosections were used for fluorescent immunohistochemistry as previously described (Joksimovic et al., 2005). Due to specific expression pattern of HOXA5 in the CNS spanning the area from the caudal hindbrain down to prevertebrae 6 (Joksimovic et al., 2005), a specific angle of sectioning was performed between a conventional coronal and transverse (horizontal) one that we term a transcoronal sectioning. Antibodies included: mouse anti-LIM1/2, mouse anti-PAX6, mouse anti-PAX7 (Developmental Studies Hybridoma Bank), guinea pig anti-LMX1B (a generous gift from

Dr. T. Jessell), rabbit anti-HOXA5 (Joksimovic et al., 2005), rabbit anti-LBX1 (a generous gift from Dr. C. Birchmeier), rabbit anti-BRN3A (a generous gift from Dr. E. Turner), rabbit anti-PHOX2B (a generous gift from Dr. J.-F. Brunet) and rabbit anti-CALBINDIN (Sigma). For triple-labeled immunofluorescence, a goat anti-rabbit Alexa Fluor 555, a goat anti-guinea pig Alexa Fluor 647, and either a goat anti-mouse Alexa Fluor 488 (Molecular Probes) or a biotinylated anti-mouse IgG-avidin conjugated FITC complex (M.O.M. kit; Vector Laboratories) were used as secondary antibodies. Sections were observed and photographed using a Zeiss Axioplan II fluorescent microscope equipped with Axiocam-digital camera. Images were further processed in Adobe Photoshop.

Results

Identification of seven dorsal hindbrain neuron populations and the initiation of HOXA5 expression in the caudal hindbrain at E10.75

We have previously shown that HOXA5 undergoes a neuronal extension into hindbrain between E10.5 and E11.5, and that HOXA5 expression was confined to postmitotic cells. In addition, the expression was detected in a subset of LIM1/2-expressing cells but not ISL1/2$^+$ cells. These data indicate that the HOXA5 hindbrain expression could be specific to the sensory system (Joksimovic et al., 2005). We wished to further define cell types expressing HOXA5 on transverse sections labeled with antibodies to dorsal interneuron markers at E10.75 (Fig. 1A-D)(Gross et al., 2002; Müller et al., 2002; Dauger et al., 2003; Ding et al., 2003; Helms and Johnson, 2003). This embryonic stage was chosen because we determined the precise temporal window of the initiation of HOXA5 expression in the caudal


LBX1 LIM1/2
LBX1 PAX7
BRN3A LIM1/2
PHOX2B LIM1/2
LMX1B
dI2
dI3a
dI3b
dI4
dI5
dI6
LBX -
LBX+
FP
vMNs
A
B
C
D
HOXA5 LIM1/2
HOXA5 PAX6
dI1
dI3a, b
E
F
G
H
PAX6
PAX7
NKX2.2
LBX1
BRN3A
LIM1/2
LMX1B
PHOX2B
ISL1/2
A⟷P
HOXA5
V0, V1
V2
vMN
psN
fp

hindbrain to be at E10.75. In addition, HOXA5 expression was dynamic, changing from dorsal to ventral along the anterior-posterior axis of the caudal hindbrain (Fig. 1E-H).

In the spinal cord, six dorsal interneuron populations (dI1-6) are defined by combinatorial expression of transcription factors whereas dI1-3 are $LBX1^-$ and dI4-6 are $LBX1^+$ populations (Gross et al., 2002; Müller et al., 2002;Helms and Johnson, 2003). Our analysis of the distribution of dorsal interneuron markers in the caudal hindbrain relative to that in the spinal cord revealed not only similarities but also notable differences. As observed in the spinal cord, three interneuron populations (dI4-6) were observed within the LBX1 expression domain: dI4 population coexpresses LBX1 and LIM1/2 but not LMX1B, dI5 population coexpresses LBX1 and LMX1B but not LIM1/2, dI6 population coexpresses LBX1 and LIM1/2 but not LMX1B including streams of dI5 (arrow) and dI6 (arrowhead) populations that appear to migrate ventrally (Fig. 1A, B). In addition, a ventral inner limit of LBX1 expressing cells was found in close proximity to that of $PAX7^+$ cells (Fig. 1B). dI5 population also coexpresses LMX1B and BRN3A (Fig. 1C). Thus, a distribution of dorsal interneuron markers within the LBX1 expression domain in the caudal hindbrain appears to be similar to that in the spinal cord (Gross et al., 2002; Müller et al., 2002;Helms and Johnson, 2003). However, within the $LBX1^-$ expression domain in the dorsal caudal hindbrain, we detected four interneuron populations as opposed to the three (dI1-3) in the spinal cord. First, we find a dI1 population that exclusively expresses BRN3A, and a dI2 population that coexpresses BRN3A and LIM1/2 but not LMX1B (Fig. 1C). Further, a dI3 population can be divided into two classes, denoted here as dI3a and dI3b. The dI3a population coexpresses LMX1B, BRN3A, LMX1B, and PHOX2B, respectively but not LIM1/2 (Fig. 1A-D). This population marks the developing nucleus of tractus solitarius

(nTS) as previously defined by a coexpression of LMX1B and PHOX2B in the dorsal caudal hindbrain (Dauger et al., 2003). In contrast, the dI3b population coexpresses BRN3A and LIM1/2, but not LMX1B, PHOX2B, or LBX1 (Fig. 1A, C, and D) representing a cell type in the caudal hindbrain not found in the corresponding region of the spinal cord (Gross et al., 2002; Müller et al., 2002;Helms and Johnson, 2003). As expected, ventral LMX1B and PHOX2B expression was detected in the floor plate and a few cells located laterally that corresponds to the developing raphe nucleus (Ding et al., 2003) and visceral motor neurons, respectively. At E10.75, HOXA5 expression was dynamic along the AP and DV axes of the caudal hindbrain consistent with, and extending, our previous report (Fig. 1E-H; Joksimovic et al., 2005). At the anterior boundary of expression, HOXA5 was detected in the DV position that corresponded to dI1 population (Fig. 1E). At a position slightly posterior to this axial level, HOXA5 retained the strong expression in dI1 region and spread medially to a subset of dI2, dI3a, dI3b, and dI4 populations (Fig. 1F) and then ventrally as the hindbrain merges into the spinal cord (Fig. 1G, H). At this AP level, a few $HOXA5^{+}$ cells were also detected in the dI5 population ($LMX1B^{+}$). No HOXA5 expression was detected immediately ventral to the dI5 population ($LIM1/2^{+}$), indicating that HOXA5 was not expressed in the dI6 population (Fig. 1G). In addition, HOXA5 immunoreactivity was found in a subset of the ventral $LIM1/2^{+}$ cells (chevron; Fig. 1G) and these cells appeared to be $PAX6^{-}$ (arrow; Fig. 1H). In summary, an analysis of the distribution of dorsal interneuron markers in the caudal hindbrain reveals that the dI3-like population is divided into two populations, which we term dI3a and dI3b, relative to that in the spinal cord (Gross et al., 2002; Müller et al., 2002;Helms and Johnson, 2003). In addition, HOXA5 initiates expression in the caudal hindbrain at E10.75, spreading the expression from the dorsal to ventral region along the AP axis and is

detected in dI1, dI2, dI3a, dI3b, dI4, and dI5 dorsal interneuron populations (summarized in Fig. 1I).

HOXA5 is essential for BRN3A expression in the dorsal lamella of the DAO

The widespread expression of HOXA5 in the caudal hindbrain at E10.75 (Fig. 1E-H), E11.5, and E13.5 (Joksimovic et al., 2005) led us to ask whether HOXA5 is expressed in this structure at 18.5, when an adult subnuclear organization of hindbrain nuclei is established. HOXA5 expression was retained in the caudal hindbrain at this stage and detected in ION (Fig. 2A). To confirm this and visualize the whole inferior olive complex, serial sections were labeled with antibodies either to BRN3A or HOXA5 (Fig. 2A, B; Xiang et al., 1996;). A comparison of HOXA5 and BRN3A expression pattern in ION revealed that the expression of HOXA5 was specific to the dorsal lamella of DAO (Fig. 2A, B). Concordant with the observed expression pattern of HOXA5, BRN3A expression was exclusively lost in this lamella in *Hoxa5-/-* embryo (arrows; Fig. 2C). Serial counterstained sections were assayed for BRN3A immunoreactivity along the entire AP extent of the ION in multiple *Hoxa5* deficient embryos, confirming a loss of BRN3A expression in this structure. In addition, immunohistochemistry with anti-CALBINDIN antibodies was also helpful in identifying inferior olive subnuclei. In wild-type mice, CALBINDIN is expressed in part of the MAO, the u-shaped region of the PO, and the ventrolateral enlargement of the DAO (Bloch-Gallego et al., 1999). No alteration in CALBINDIN immunoreactivity was observed in *Hoxa5* deficient embryos (data not shown).

Therefore, these findings demonstrate that HOXA5 expression is specific to the dorsal lamella of DAO within the inferior olivary complex and that the HOXA5 protein is

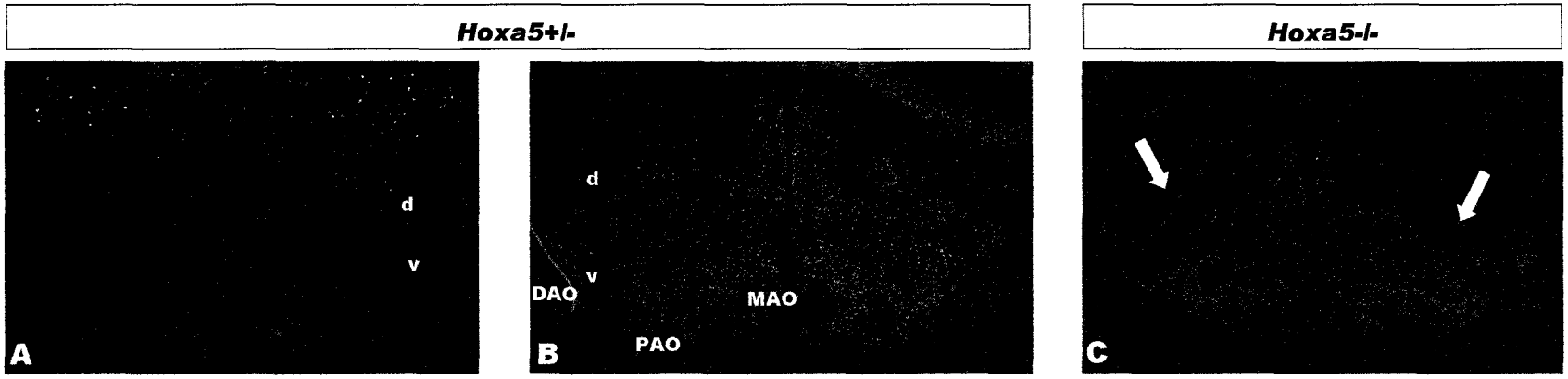


Fig. 2. HOXA5 is required for BRN3A expression in dorsal lamella of DAO at E18.5. (A) HOXA5 expression (magenta) is confined to dorsal lamella (d) of dorsal accessory olive (DAO). (B) All three subnuclei of ION can be clearly visualized by BRN3A immunohistochemistry (magenta). (C) Arrows indicate a loss of BRN3A expression in dorsal lamella of DAO in *Hoxa5* deficient embryos. MAO, medial accessory olive; PAO, principal accessory olive; v, ventral lamella of DAO.

required for BRN3A expression in this structure.

HOXA5 is dispensable for BRN3A and LIM1/2 expression in the inferior olive neurons between E11.5-13.5

To gain insight into the loss of BRN3A expression in the dorsal lamella of DAO observed at E18.5, we examined earlier embryonic profile of BRN3A in the developing ION between E11.5-13.5, a time window shown to be important for migration and settling pattern of inferior olive neurons (Bloch-Gallego et al., 1999).

At E11.5, in sections double-stained with antibodies to BRN3A and LIM1/2, a population of cells coexpressing these two proteins can be seen at the lateral edge of the dorsal hindbrain (arrow; Fig. 3A). This population appears to migrate ventrally at E12.5 (arrow; Fig. 3B) and settle in a club-shaped domain close to the floor plate at E13.5 (arrow; Fig. 3C). This is the recognizable formation of newly arrived inferior olive neurons (Bloch-Gallego et al., 1999), while other cells coexpressing BRN3A and LIM1/2 seemed to be outside the olivary territory (arrowhead; Fig. 3C). At E11.5 and E12.5, HOXA5 was detected in a subset of medio-lateral and ventral LIM1/2^{+} cells of the caudal hindbrain (Joksimovic et al., 2005; data not shown). At E13.5, HOXA5 immunoreactivity partially overlapped with that of LIM1/2 (arrow; Fig. 3D) in the ventral region close to the floor plate corresponding to the clustered inferior olive neurons. In addition, HOXA5 expression was restricted to the caudal region of ION while BRN3A and LIM1/2 were expressed throughout the rostro-caudal extent of this nucleus (Fig. 3E, F). Despite specific HOXA5 expression in developing ION, BRN3A and LIM1/2 expression patterns were intact in *Hoxa5-/-*embryos at E13.5 as well as E11.5-12.5 (data not shown). HOXA5 immunoreactivity was also detected in

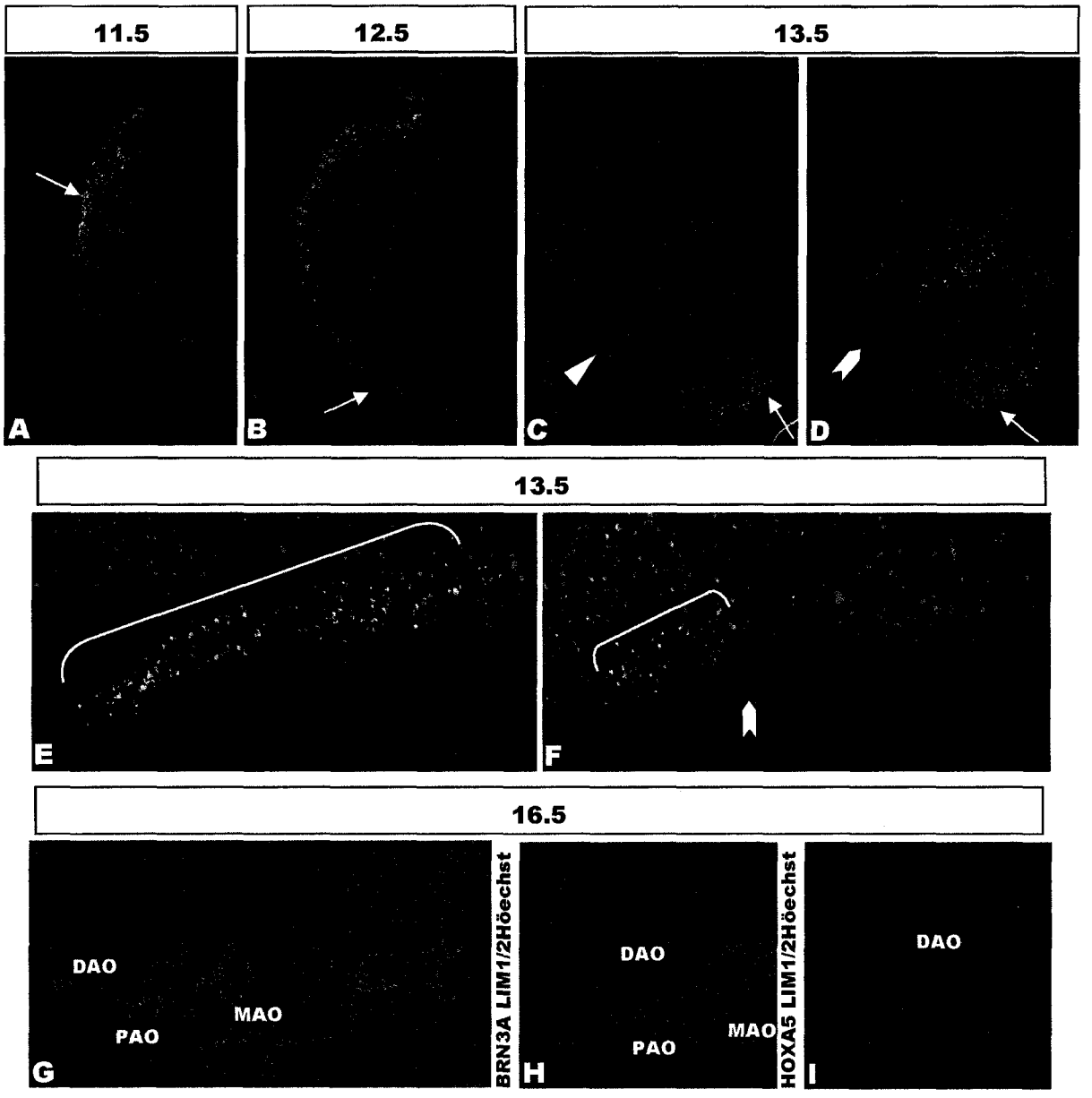


Fig. 3. HOXA5, BRN3A, and LIM1/2 expression in developing inferior olive neurons. (A-D) Temporal expression pattern of BRN3A and LIM1/2 in cells that appear to mark migratory (arrows in A and B; arrowhead in C) and settled neurons of ION (arrow in C) in wild-type embryos. HOXA5 is expressed in ION (arrow) and superficial migration (chevron). (E, F) Anterior-posterior expression pattern of HOXA5, BRN3A, and LIM1/2 in newly settled neurons of ION in *Hoxa5*+/- embryos. BRN3A^{+}/LIM1/2^{+} cells can be seen throughout rostro-caudal axis of ION while HOXA5 expression is restricted to the caudal region of the nucleus (brackets) and found in superficial migration (chevron). (G-I) Initiation of adult lamination in ION. In sections prepared from *Hoxa5*+/- embryos, medial accessory olive (MAO), principal accessory olive (PAO), and dorsal accessory olive (DAO) can be visualized by BRN3A expression. HOXA5 immunoreativity appears to be specific to DAO.

marginal stream of cells located laterally to the outer edge of hindbrain (chevron; Fig. 3D). The expression in these cells can be also clearly seen in the sagittal section double-stained for HOXA5 and LIM1/2 (chevron; Fig. 3F). In addition, neither BRN3A nor LIM1/2 were detected in the corresponding region (Fig. 3E, F). This marginal stream of cells likely represents a superficial neuronal migration judging by its position relative to the ION (Cambronero and Puelles, 2000). In summary, HOXA5 appears not to be required for expression of transcription factors BRN3A and LIM1/2 that mark newly arrived inferior olive neurons in the ventral hindbrain at E13.5.

HOXA5 is not required to maintain BRN3A and LIM1/2 expression in the inferior olive neurons at E16.5

In mice, the ION appears as a laminated structure at E16.5 (Bloch-Gallego et al., 1999). Accordingly, we examined the expression pattern of BRN3A and LIM1/2 at this stage to determine if HOXA5 plays a role in maintaining the observed transcriptional code of the ION relative to the initiation of lamination of this nucleus. Serial sections of *Hoxa+/-* embryos stained for BRN3A and LIM1/2 antibodies revealed no LIM1/2 expression in ION. In addition, a strong BRN3A immunoreactivity was detected in MAO, PAO, and DAO (Fig. 3G). However, at this stage DAO does not appear to be further compartmentalized into dorsal and ventral lamella as seen at E18.5 (Fig. 2B). In a slightly posterior section relative to that shown in Fig. 3G, a similar distribution of $BRN3A^{+}$ cells was seen in ION (Fig. 3H). HOXA5 expression was maintained in this nucleus and within the ION appeared to be specific to DAO (Fig. 3I). Again, in spite of persistent expression of HOXA5 in the developing ION, no differences in BRN3A olivary expression were found in *Hoxa5-/-*

embryos (data not shown). Taken together, HOXA5 appears to be dispensable to maintaining the transcriptional code of ION at E16.5. Further, a fully laminated ION morphology is achieved between E16.5-18.5.

Discussion

In this study, we show, to date, the first evidence for the role of any *Hox5* paralogue in development of the caudal hindbrain. Our findings reveal a number of important points.

First, a combinatorial transcriptional code in specification of the dorsal neuron population in caudal hindbrain appears to be different to that seen in the spinal cord (Gross et al., 2002; Müller et al., 2002;Helms and Johnson, 2003) as indicated by previous studies (Engelkamp et al., 1999; Qian et al., 2001; Dauger et al., 2003; Ding et al., 2003). Here we demonstrate that the dI3 population can be further divided into two classes that we term dI3a and dI3b. The dI3a cells correspond to the molecular signature of the developing nucleus of tractus solitarius (nTS) as they coexpress LMX1B and PHOX2B (this study; Dauger et al., 2003) and RNX (Qian et al., 2001). HOXA5 was detected in a subset of $LMX1B^+$ cells of the dI3a population at 10.75-13.5 (Fig. 1F-H; data not shown) indicating that HOXA5 may be involved in molecular specification of the developing nTS. Our immunohistochemical analysis using LMX1B, PHOX2B, and tyrosine hydroxylase (TH) antibodies revealed that HOXA5 is neither required nor sufficient for the molecular signature of nTS cells and the expression of TH in the medullary, A2C2 catecholaminergic center (data not shown). Although ectopic expression of HOXA5 protein in the dorsal spinal cord of *Hoxa5SV2* mutant embryos (Krieger et al., 2004) induced a few ectopic $PHOX2B^+$ cells only at forelimb spinal levels, these cells appeared not to express TH (Fig. 4). A compensatory mechanism of

Hox5 paralog genes and/or other *Hox* genes with overlapping expression domains may provide an explanation for our results. Alternatively, HOXA5 may function in the

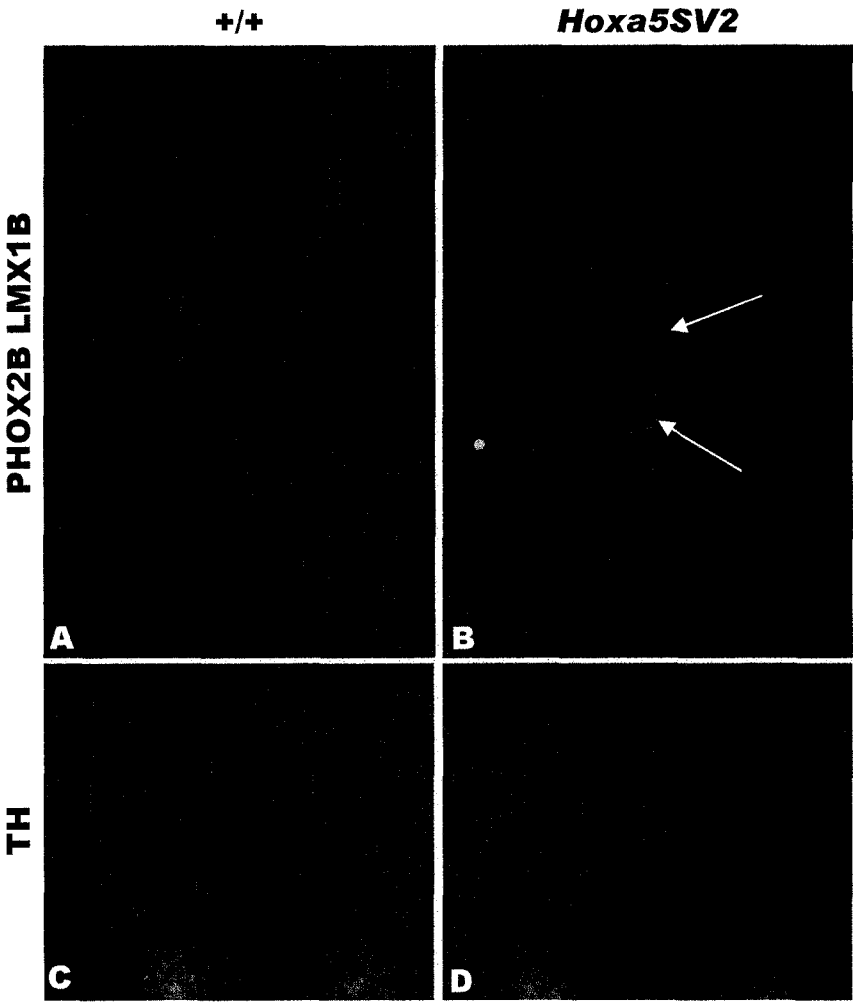


Fig. 4. HOXA5 induces PHOX2B but not tyrosine hydroxylase (TH) expression at forelimb spinal levels. (A-D) Serial transverse sections of the spinal cord were labeled with PHOX2B (red), LMX1B (green) (A, B), and TH antibodies (C, D) in wild-type (A, C) and *Hoxa5SV2* embryos (B, D) at E10.5. Arrows indicate ectopic PHOX2B^{+} cells whereas no change in TH immunoreactivity was observed in *Hoxa5SV2* mutant spinal cords (B, D) in comparison to those in wild-type (A, C).

catecholaminergic pathway down-stream from TH, as PHOX2 and HOXA5 proteins can interact with the human norepinephrine transporter gene promoter *in vitro* (Kim et al., 2002). A developmental fate of dI3b cells coexpressing BRN3A and LIM1/2, but not LMX1B, PHOX2B, or LBX1 (Fig. 1A, C, and D) is unclear since, using a panel of nine antibodies (Fig. 1), we could not distinguish this population from dI2 expressing the same combinatorial transcriptional code. Within the LBX1 expression domain, three interneuron populations (dI4-6) were detected as reported for the spinal cord (Gross et al., 2002; Müller et al., 2002; Helms and Johnson, 2003).

Second, HOXA5 initiates the expression from the spinal cord to its definitive anterior limit of expression in caudal hindbrain at E10.75, and appears to be excluded from the dI6 populations (Fig. 1). The onset of HOXA5 expression in the caudal hindbrain may be important for cellular specification of several nuclei that initiate their complex migration in this temporal window (Engelkamp et al., 1999; Ding et al., 2003).

Third, we demonstrate that the ION could be transiently defined by a combinatorial expression of BRN3A and LIM1/2 transcription factors that may belong to either dI2 or dI3b populations. Between E11.5-12.5, $BRN3A^{+}/LIM1/2^{+}$ cells appear to migrate from dorsal to the ventral hindbrain consistent with the known timing of inferior olive neuron migration in mice (Fig. 3A, B; Bloch-Gallego et al., 1999). At E13.5, cells coexpressing these two transcription factors were detected in the ventral hindbrain close to the floor plate, a region that corresponds to the DV position of clustered inferior olive neurons (Fig. 3C, E). At the same embryonic age, despite a region-specific expression of HOXA5 in ION (Fig. 3F), the protein appears to be dispensable for BRN3A and LIM1/2 expression in newly settled inferior olive neurons. At E16.5, LIM1/2 is down-regulated while BRN3A retained

expression in the ION (Fig. 3G, H). Further, we show that a lamination of the ION is elaborated between E16.5-18.5 (compare Fig. 2B and Fig. 3G). Most importantly, we show that HOXA5 is essential to maintain the expression of BRN3A in the dorsal lamella of the DAO at E18.5 (Fig. 2). In the ION, this late requirement of HOXA5 for BRN3A expression and cessation of LIM1/2 immunoreactivity at E16.5 implies that a combinatorial transcriptional code could be temporally switched from ION migration (E11.5-13.5), via climbing fiber axonogenesis to cerebellum (E15.5-birth) to early transient synaptogenesis (P0-P5) and climbing fiber maturation including synaptic stabilization (P7-P15)(Sherrard and Bower, 2002). In addition, expression of HOXA5 in the dorsal lamella of the DAO (Fig. 2) and specific projections of each of the inferior olivary subnuclei to regions of the cerebellum (Sugihara and Shinoda, 2004) suggest that HOXA5 may have a function in establishment of early synaptogenesis, climbing fiber maturation and synaptic stabilization (P0-P15).

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References

Altman, J., Bayer, S., A., 1987. Development of the precerebellar nuclei in the rat: II. The intramural olivary migratory stream and the neurogenetic organization of the inferior olive. J. Comp. Neurol. 257, 490 –512.

Aubin, J., Lemieux, M., Tremblay, M., Bérard, J., Jeannotte, L., 1997. Early postnatal lethality in *Hoxa-5* mutant mice is attributable to respiratory tract defects. Dev. Biol. 192, 432-445.

Aubin, J., Lemieux, M., Tremblay, M., Behringer, R.R., Jeannotte, L., 1998. Transcriptional interferences at the *Hoxa4/Hoxa5* locus: Importance of correct *Hoxa5* expression for the proper specification of the axial skeleton. Dev. Dyn. 212, 141-156.

Aubin, J., Chailler, P., Ménard, D., Jeannotte, L., 1999. Loss of *Hoxa5* gene function in mice perturbs intestinal maturation. Am. J. Physiol. 277, C965–C973.

Aubin, J., Lemieux, M., Moreau, J., Lapointe, J., Jeannotte, L., 2002a. Cooperation of *Hoxa5* and *Pax1* genes during formation of the pectoral girdle. Dev. Biol. 244, 96-113.

Aubin, J., Déry, U., Lemieux, M., Chailler, P., Jeannotte L., 2002b. Stomach regional specification requires *Hoxa5*-driven mesenchymal-epithelial signaling. Development 129, 4075-4087.

Azizi, S.A., Woodward, D.J., 1987. Inferior olivary nuclear complex of the rat: morphology and comments on the principles of organization within the olivocerebellar system. J. Comp. Neurol. 22, 467-84.

Bell, E., Wingate, R.J., Lumsden, A., 1999. Homeotic transformation of rhombomere identity after localized Hoxb1 misexpression. Science 284, 2168-2171.

Bloch-Gallego, E., Ezan, F., Tessier-Lavigne, M., Sotelo, C., 1999. Floor plate and netrin-1 are involved in the migration and survival of inferior olivary neurons. J. Neurosci. 19, 4407-4420.

Brend, T., Gilthorpe, J., Summerbell, D., Rigby, P.W., 2003. Multiple levels of transcriptional and post-transcriptional regulation are required to define the domain of *Hoxb4* expression. Development 130, 2717-2728.

Cambronero, F., Puelles, L., 2000. Rostrocaudal nuclear relationships in the avian medulla oblongata: a fate map with quail chick chimeras. J. Comp. Neurol. 427, 522-545.

Carpenter, E.M., Goddard, J.M., Davis, A.P., Nguyen, T.P., Capecchi, M.R., 1997. Targeted disruption of Hoxd-10 affects mouse hindlimb development. Development 124, 4505-4514.

Dauger, S., Pattyn, A., Lofaso, F., Gaultier, C., Goridis, C., Gallego, J., Brunet, J-F., 2003. Phox2b controls the development of peripheral chemoreceptors and afferent visceral pathways. Development. 130, 6635-6642.

Ding, Y.Q., Marklund, U., Yuan, W., Yin, J., Wegman, L., Ericson, J., Deneris, E., Johnson, R.L., Chen, Z.F., 2003. Lmx1b is essential for the development of serotonergic neurons. Nat. Neurosci. 6, 933-938.

Dasen, J.S., Liu, J-P., Jessell, T.M., 2003. Motor neuron columnar fate imposed by sequential phases of Hox-c activity. Nature 425, 926-933.

Dony, C., Gruss, P., 1987. Specific expression of the *Hox 1.3* homeo box gene in murine embryonic structures originating from or induced by the mesoderm. EMBO J. 6, 2965-2975.

Engelkamp, D., Rashbass, P., Seawright, A., van Heyningen, V., 1999. Role of Pax6 in development of the cerebellar system. Development 126, 3585-3596.

Gaufo, G.O., Flodby, P., Capecchi, M.R., 2000. Hoxb1 controls effectors of sonic hedgehog and Mash1 signaling pathways. Development 127, 5343-5354.

Gaufo, G.O., Thomas, K.R., Capecchi, M.R., 2003. Hox3 genes coordinate mechanisms of genetic suppression and activation in the generation of branchial and somatic motoneurons. Development 130, 5191-5201.

Gaufo, G.,O., Wu, S., Capecchi, M.R., 2004. Contribution of Hox genes to the diversity of the hindbrain sensory system. Development 131, 1259-1266.

Goddard, J.M., Rossel, M., Manley, N.R., Capecchi, M.R., 1996. Mice with targeted disruption of Hoxb-1 fail to form the motor nucleus of the VIIth nerve. Development 122, 3217-3228.

Gaunt, S.J., Sharpe, P.T., Duboule, D., 1988. Spatially restricted domains of homeo-gene transcripts in mouse embryos: relation to a segmented body plan. Development 104, 169-179.

Gaunt, S.J., Coletta, P.L., Pravtcheva, D., Sharpe, P.T., 1990. Mouse *Hox3.4*: Homeobox sequence and embryonic expression patterns compared with other members of the *Hox* gene network. Development 109, 329-339.

Gross, M., Dottori, M., Goulding, M., 2002. *Lbx1* specifies somatosensory association interneurons in the dorsal spinal cord. Neuron 34, 535-549.

Guidato, S., Prin, F., Guthrie, S., 2003. Somatic motoneurone specification in the hindbrain: the influence of somite-derived signals, retinoic acid and Hoxa3. Development 130, 2981-2996.

Harlow, E., Lane, D., 1988. Antibodies: A laboratory manual, first ed. Cold Spring Harbor Laboratory Press, New York.

Helms, A., Johnson, J., 2003. Specification of dorsal spinal cord interneurons. Curr. Opin. Neurobiol.13, 42-49.

Hogan, B., Constantini, F., Beddington, R., Lacy, E., 1994. Manipulating the mouse embryo: A laboratory manual, second ed. Cold Spring Harbor Laboratory Press, New York.

Jeannotte, L., Lemieux, M., Charron, J., Poirier, F., Robertson, E.J., 1993. Specification of axial identity in the mouse: role of the *Hoxa-5 (Hox1.3)* gene. Genes Dev. 7, 2085-2096.

Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev. Genet. 1, 20-29.

Joksimovic, M., Jeannotte, L., Tuggle, C.K., 2005. Dynamic expression of murine HOXA5 protein in the central nervous system (in press; Gene Expression Patterns)

Kaufman, M.H., 1992. The atlas of mouse development, first ed. Academic Press, San Diego.

Krieger, K.E., Abbott, M.A., Joksimovic, M., Lueth, P.A., Sonea, I.M., Jeannotte, L., Tuggle, C.K., 2004. Transgenic mice ectopically expressing HOXA5 in the dorsal spinal cord show structural defects of the cervical spinal cord along with sensory and motor defects of the forelimb. Brain Res. Dev. Brain Res. 150, 125-139.

Krumlauf, R., 1994. *Hox* genes in vertebrate development. Cell 78, 191-201.

Larochelle, C., Tremblay, M., Bernier, D., Aubin, J., Jeannotte, L., 1999. Multiple *cis*-acting regulatory regions are required for the restricted spatio-temporal *Hoxa5* gene expression. Dev. Dyn. 214, 127-140.

Meunier, D., Aubin, J., Jeannotte L., 2003. Perturbed thyroid morphology and transient hypothyroidism symptoms in *Hoxa5* mutant mice. Dev. Dyn. 227, 367-378.

Moran-Rivard, L., Kagawa, T., Saueressig, H., Gross, M.K., Burrill, J., Goulding, M., 2001. *Evx1* is a postmitotic determinant of V0 interneuron identity in the spinal cord. Neuron 29, 385–399.

Müller, T., Brohmann, H., Pierani, A., Heppenstall, P.A., Lewin, G.R., Jessell, T.M., Birchmeier, C., 2002. The homeodomain factor Lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron 34, 551-562.

Odenwald, W.F., Taylor, C.F., Palmer-Hill, F.J., Friedrich, V.Jr., Tani, M., Lazzarini, R.A., 1987. Expression of a homeo domain protein in noncontact-inhibited cultured cells and postmitotic neurons. Genes Dev. 1, 482-496.

Odenwald, W.F., Garbern, J., Arnheiter, H., Tournier-Lasserve, E., Lazzarini, R.A., 1989. The *Hox1.3* homeo box protein is a sequence-specific DNA-binding phosphoprotein. Genes Dev. 3, 158-172.

Oosterveen, T., Niederreither, K., Dolle, P., Chambon, P., Meijlink, F., Deschamps, J., 2003. Retinoids regulate the anterior expression boundaries of 5' *Hoxb* genes in posterior hindbrain. EMBO J. 22, 262-269.

Oosterveen, T., Meijlink, F., Deschamps, J., 2004. Expression of Retinaldehyde dehydrogenase II and sequential activation of *5' Hoxb* genes in the mouse caudal hindbrain. Gene Expr. Patterns 4, 243-247.

Pierani, A., Brenner-Morton, S., Chiang, C., Jessell, T.M., 1999. A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell 97, 903-115.

Pierani, A., Moran-Rivard, L., Sunshine, M.J., Littman, D.R., Goulding, M., Jessell, T.M., 2001. Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. Neuron 29, 367-384.

Qian, Y., Fritzsch, B., Shirasawa, S., Chen, C.L., Choi, Y., Ma, Q., 2001. Formation of brainstem (nor)adrenergic centers and first-order relay visceral sensory neurons is dependent on homeodomain protein Rnx/Tlx3. Genes Dev. 15, 2533-2545.

Sanlioglu, S., Zhang, X., Baader, S.L., Oberdick, J., 1998. Regulation of a Purkinje cell-specific promoter by homeodomain proteins: repression by engrailed-2 vs. synergistic activation by Hoxa5 and Hoxb7. J. Neurobiol. 36, 559-571.

Sherrard, R., M., Bower, 2002. Climbing fiber development: do neurotrophins have a part to play? The Cerebellum, 1: 265-276

Sockanathan, S., Jessell, T.M., 1998. Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. Cell 94, 503-514.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A., Krumlauf, R., 1996. Altered segmental identity and abnormal migration of motor neurons in mice lacking Hoxb-1. Nature 384, 630-634.

Sugihara, I., Shinoda, Y., 2004. Molecular, topographic, and functional organization of the cerebellar cortex: a study with combined aldolase C and olivocerebellar labeling. J. Neurosci. 24, 8771-8785.

Tani, M., Odenwald, W.F., Lazzarini, R.A., Friedrich, V.L.Jr., 1989. Progressive restriction in the distribution of the *Hox-1.3* homeodomain protein during embryogenesis. J. Neurosci. Res. 24, 457-469.

Tiret, L., le Mouellic, H., Maury, M., Brulet, P., 1998. Increased apoptosis of motoneurons and altered somatotopic maps in the brachial spinal cord of Hoxc-8-deficient mice. Development 125, 279-291.

Varela-Echavarría, A., Pfaff, S.L., Guthrie, S., 1996. Differential expression of LIM homeobox genes among motor neuron subpopulations in the developing chick brain stem. Mol. Cell. Neurosci. 8, 242-257.

Wall, N.A., Jones, M., Hogan, B.L.M., Wright, C.V.E., 1992. Expression and modification of Hox 2.1 protein in mouse embryos. Mech. Dev. 37, 111-120.

Xiang, M., Gan, L., Zhou, L., Klein, W.H., Nathans, J., 1996. Targeted deletion of the mouse POU domain gene Brn-3a causes selective loss of neurons in the brainstem and trigeminal ganglion, uncoordinated limb movement, and impaired suckling. Proc. Natl. Acad. Sci. USA 93, 11950-11955.

CHAPTER V. General Conclusions

Summary of Work Presented in Thesis

Murine *Hoxa5* gene plays a crucial role in proper embryonic patterning of the vertebrate body axes. Transcription of this gene produces a complex pattern of multiple mRNAs. One of these transcripts, the 1.8kb, appears to be specific to several mesoderm-derived structures affected by the *Hoxa5* mutation. Although *Hoxa5* is expressed in the spinal cord and hindbrain, no central nervous system (CNS) phenotype has yet been reported in *Hoxa5* deficient animals.

To verify the functional significance of the 1.8kb transcript in development and to correlate the presence of the HOXA5 protein and RNAs we developed HOXA5-specific antibodies and analyzed the protein immunolocalization during embryogenesis. Our immunohistochemical analyses revealed that the HOXA5 protein expression pattern corresponded closely to that of the 1.8kb transcript, confirming and extending the importance of this transcript in the development of mesoderm-derived structures. In the CNS, the anterior limit of HOXA5 expression expended from the cervical spinal cord to the caudal hindbrain between embryonic day (E)10.5 and E11.5. Moreover, the HOXA5 expression pattern was region-specific, spanning the area from the hypoglossal nucleus in the caudal hindbrain to the ventral cervical/brachial spinal cord, and was more restricted than the *RNA* expression pattern obtained using *in situ* analysis. The protein expression was also temporally dynamic and detected in the superficial region of the dorsal spinal cord at E15.5. In addition, the protein was found predominantly in interneurons in the hindbrain while the

expression was detected in both motor neuron and interneuron populations in the spinal cord. Analysis of HOXA5 expression pattern in the CNS provided a necessary basis for future work to gain insight to the function of the protein in the CNS development. These studies are presented in Chapter III as the first author manuscript in press in *Gene Expression Patterns* (Joksimovic et al., 2005) and Appendix II as a co-authored manuscript in preparation (Coulombe et al., 2005). The thesis author provided an immunohistochemical analysis of the HOXA5 expression pattern in mesoderm-derived structures at E12.5 and E13.5.

We next focused on a complex HOXA5 expression pattern in the caudal hindbrain in an attempt to gain insight to the function of HOXA5 in this complex system. As stated above, HOXA5 immunohistochemical analysis showed that the protein expression extended into the caudal hindbrain within a narrow time window, between E10.5 and E10.75. We next wished to extend our previous findings and further define hindbrain cell types expressing HOXA5. To do so, transverse sections were labeled with a panel of antibodies to dorsal neuron markers at E10.75. Defined by combinatorial expression of transcription factors, seven dorsal interneuron (dI) populations (dI, dI2, dI3a, dI3b, dI4, dI5, and dI6) were detected in caudal hindbrain as oppose to six in the spinal cord (dI1-6)(Gross et al., 2002; Müller et al., 2002; Helms and Johnson, 2003). HOXA5 immunoreactivity was detected in dI1, dI2, dI3a, dI3b, dI4, and dI5 dorsal interneuron populations. The expression of HOXA5 was retained in the caudal hindbrain throughout development. A day before birth, at E18.5, the HOXA5 expression corresponded to several clustered neuronal cell bodies referred to as nuclei. In one of them, the inferior olivary nucleus (ION), HOXA5 expression was restricted to dorsal lamella of the dorsal accessory olive (DAO) subnucleus. We further showed that the ION could be transiently defined by a combinatorial expression of BRN3A and LIM1/2

transcription factors. Moreover, HOXA5 was required to maintain the expression of BRN3A in the dorsal lamella of DAO. However, HOXA5 is dispensable for the transcriptional code of ION up to embryonic day (E)16.5. This result may be due to compensatory effect of *Hox5* paralogs and/or other *Hox* genes with overlapping expression domains. Taken together, our findings indicates that HOXA5 plays an important role in maintaining the transcriptional code of the ION that may affect postnatal establishment of connectivity, maturation or synaptic stabilization of ION projections (climbing fibers) to the cerebellum. These studies are presented in Chapter IV as the first author manuscript intended for submission to *Developmental Biology* (Joksimovic et al., 2005).

Additionally, a substantial work has been performed to determine a possible function of HOXA5 in the other hindbrain structure, nucleus of tractus solitarius (nTS), where HOXA5 expression is observed. This nucleus is an important autonomic relay center that receives sensory information from a variety of internal organs and is involved in neuronal control of breathing. During development, nTS neurons coexpress LMX1B and PHOX2B transcription factors in the dorsal caudal hindbrain (Dauger et al., 2003). Later in development, the expression of tyrosine hydroxylase is initiated within the nTS, forming the A2C2 catecholaminergic center. An input from this center to the respiratory muscles via the ventral hindbrain decreases the frequency of respiration. A disruption of the *Hoxa5* gene results in impaired tracheal and lung morphogenesis that is likely to be the cause of early postnatal lethality of *Hoxa5* mutant mice (Aubin et al., 1997). However, surviving *Hoxa5-/-* animals show respiratory adaptations including a higher breathing frequency and overall minute ventilation that likely compensates for impaired morphogenesis of the respiratory tract during development (Kinkead et al., 2004). In addition, potential recognition sites for

HOXA5 shared across human, mouse, and rat has been reported in the tyrosine hydroxylase promoter (Kessler et al., 2003).

Initially, our immunohistochemical analysis of wild-type embryos revealed that HOXA5 was expressed in a subset of $LMX1B^+$ cells. We then asked if HOXA5 is required for molecular specification of nTS and tyrosine hydroxylase (TH) expression in A2C2 catecholaminergic center. Our data indicated that HOXA5 is dispensable for the above-mentioned developmental events as assayed by LMX1B, PHOX2B, and TH immunohistochemistry on sections prepared from multiple wild-type and *Hoxa5-/-* embryos at several stages of development. We also asked if HOXA5 is sufficient to induce expression of LMX1B, PHOX2B, and TH in embryos of the *Hoxa5SV2* line that ectopically expresses HOXA5 in the dorsal spinal cord (Krieger et al., 2004). No differences in LMX1B and TH immunoreactivity were seen on the sections prepared from multiple wild-type and *Hoxa5SV2* embryos at E10.5 and E12.5, although we detected a few ectopic $PHOX2B^+$ cells that appeared not to express TH.

In summary, our data indicate that HOXA5 is neither required nor sufficient for molecular specification of nTS and the expression of TH in A2C2 catecholaminergic center. A redundant function of *Hox5* paralog genes may provide an explanation for our results. Alternatively, HOXA5 may function in catecholaminergic pathway down-stream from TH as PHOX2 and HOXA5 proteins can interact with the human norepinephrine transporter gene promoter *in vitro* (Kim et al., 2002).

The thesis author contributed to initial characterization of the *Hoxa5SV2* mouse line that led to a co-authored publication in *Developmental Brain Research* (Krieger et al, 2004) that is the first, published use of HOXA5 antibodies. The thesis author also contributed to a

co-authored manuscript to be resubmitted after a revision to *Developmental Brain Research* (Abbott et al., 2005) providing an immunohistochemical analysis of the BRN3A, LBX-1, and LIM1/2 expression patterns in wild-type and *Hoxa5SV2* embryonic spinal cords.

References

Aubin, J., Lemieux, M., Tremblay, M., Bérard, J., Jeannotte, L., 1997. Early postnatal lethality in *Hoxa-5* mutant mice is attributable to respiratory tract defects. Dev. Biol. 192, 432-445.

Abbott, M.A., **Joksimovic, M.,** Tuggle, C.K., 2005. Ectopic HOXA5 expression results in abnormal differentiation, migration and p53-independent cell death of superficial dorsal horn neurons (to be resubmitted after revision to Developmental Brain Research)

Coulombe, Y., Lemieux, M., Moreau, J., Tabariès, S., Larochelle, C., **Joksimovic, M.,** Tuggle, C.K., Aubin J., Jeannotte, L., 2005. Different promoters and alternative splicing: transcriptional complexity at the murine *Hoxa5* locus (manuscript in preparation)

Dauger, S., Alexandre Pattyn, A., Lofaso, F., Gaultier, C., Goridis, C., Gallego, J., Brunet, J.-F., 2003. Phox2b controls the development of peripheral chemoreceptors and afferent visceral pathways. Development 130, 6635-6642.

Gross, M., Dottori, M., Goulding, M., 2002. *Lbx1* specifies somatosensory association interneurons in the dorsal spinal cord. Neuron 34, 535-549.

Helms, A., Johnson, J., 2003. Specification of dorsal spinal cord interneurons. Curr. Opin. Neurobiol.13, 42-49.

Joksimovic, M., Jeannotte, L., Tuggle, C.K., 2005. Dynamic expression of murine HOXA5 protein in the central nervous system (in press; Gene Expression Patterns)

Joksimovic, M., Abbott, M.A., Tuggle, C.K., 2005. Role of murine HOXA5 in development of the inferior olivary nucleus (to be submitted to Developmental Biology)

Kessler, M., A., Yang, M., Gollomp, K., L., Jin, H., Iacovitti, L., 2003. The human tyrosine hydroxylase gene promoter. Mol. Brain Res. 112, 8–23.

Kim, C.-H., Hwang, D.-Y., Jae-Joon Park, J.-J., Kim, K.-S., 2002. A proximal promoter domain containing a homeodomain-binding core motif interacts with multiple

transcription factors, including HoxA5 and Phox2 proteins, and critically regulates cell type-specific transcription of the human norepinephrine transporter gene. J. Neurosci. 22, 2579–2589.

Kinkead, R., Leblanc, M., Gulemetova, R., Lalancette-Hébert, M., Lemieux, M., Mandeville, I., Jeannotte, L., 2004. Respiratory adaptations to lung morphological defects in adult mice lacking *Hoxa5* gene function. Pediatr. Res. 56, 553-562.

Krieger, K.E., Abbott, M.A., **Joksimovic, M.,** Lueth, P.A., Sonea, I.M., Jeannotte, L., Tuggle, C.K., 2004. Transgenic mice ectopically expressing HOXA5 in the dorsal spinal cord show structural defects of the cervical spinal cord along with sensory and motor defects of the forelimb. Brain Res. Dev. Brain Res. 150, 125-139

Müller, T., Brohmann, H., Pierani, A., Heppenstall, P.A., Lewin, G.R., Jessell, T.M., Birchmeier, C., 2002. The homeodomain factor Lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron 34, 551-562.

APPENDICES

APPENDIX I. Development of HOXA5-specific antibodies

HOXA5 polyclonal antibodies were generated against a bacterially produced glutathione-S-transferase (GST)/mouse HOXA5 exon I antigen. The antigen was injected in two rabbits followed by the purification of the crude sera using three consecutive depletions and one affinity purification (Fig. 1).

Antigen production

E. coli $DH_5\alpha$ cells transformed with the pLJ51 vector were generously provided by Dr. Lucie Jeannotte. The vector contained a 284 bp *Hoxa5* fragment of the exon I inserted into the BamHI site at the 3' end of the GST coding region of the pGEX–2T vector (Amersham) encoding amino acids 61-151 of the predicted HOXA5 protein (Odenwald et al., 1987; Fig. 1). The correct *Hoxa5* sequence and reading frame in the fusion construct were confirmed by sequencing carried out by DNA facility at Iowa State University.

GST/HOXA5 exon I fusion protein was induced by isopropyl thio-galactoside (IPTG) to 0.2mM final concentration for 3 hours (Fig. 1). Total proteins were isolated by sonication and the concentration determined by the Bradford assay. The fusion protein was affinity purified by incubation with Glutathione-Sepharose 4B (Amersham) eluting with 10 mM glutathione in 50 mM Tris-HCl, pH 8.5 (Fig. 1). In addition, the GST/HOXA5 exon I fusion protein was digested on the beads by thrombin protease according to manufacturer's

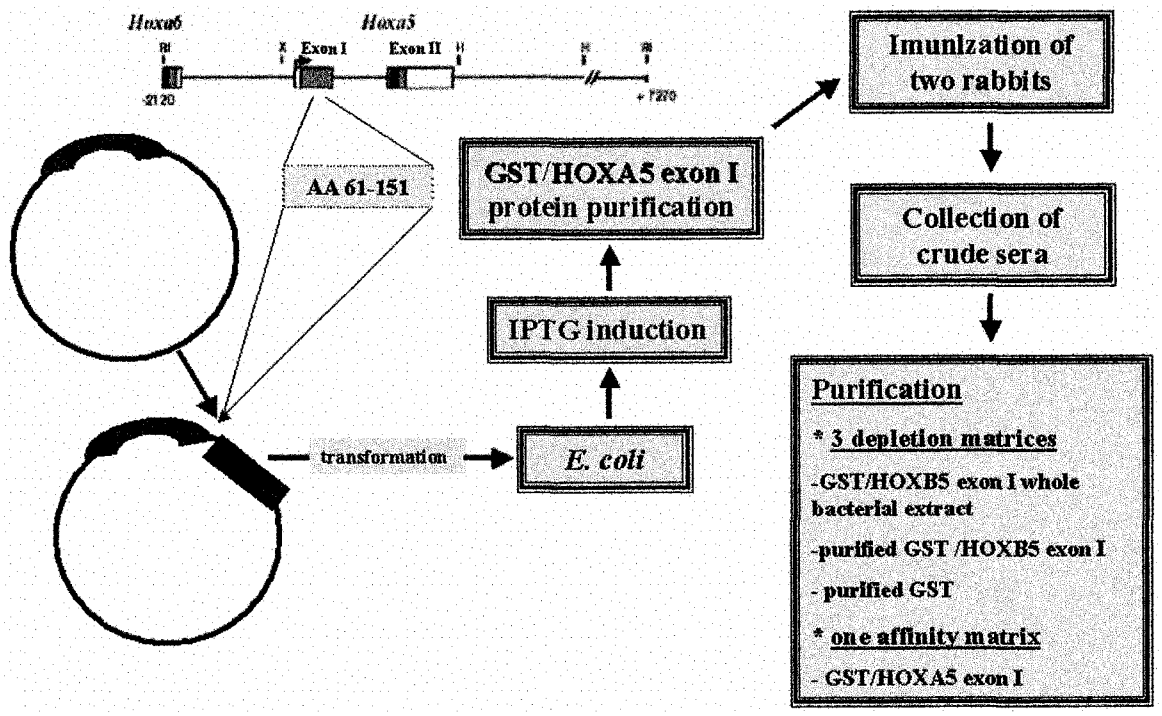

Fig. 1. Schematic representation of the development of HOXA5-specific antibodies. The antibodies were generated against a bacterially produced GST/HOXA5 exon 1 fusion protein and subsequently purified using various protein extracts coupled to CNBr-preactivated Sepharose (see text for detailed discussion)

instructions (Amersham). The same procedure was used to induce and isolate GST and GST/HOXB5 exon I proteins. All three purified proteins were stored at -20^0C.

To prepare *E. coli* cells to express the GST protein, $DH_5\alpha$ cells were made competent by $CaCl_2$ treatment and transformed by heat-shock procedure using pGEX 4T-2 vector (Amersham). To prepare *E. coli* cells to express the GST/HOXB5 exon I fusion protein the following procedure was used: a) a pGEX-3X vector containing a portion of the *Hoxb5* exon I that corresponded to the similar region of the *Hoxa5* exon I used to prepare HOXA5 antigen was isolated from *E. coli* JM109 cells (Wall et al., 1992; generously provided by Dr. Brigit Hogan) b) the vector was introduced into $DH_5\alpha$ cells using the procedure as described for the GST preparation.

Immunization

Immunization of two rabbits with GST/HOXA5 exon I fusion protein was carried out by Hybridoma Facility at Iowa State University. The sera were collected and used for a subsequent purification.

Antibody purification

HOXA5 antibodies were purified by three consecutive depletions followed by affinity purification of the crude serum (final bleed from one rabbit) on columns containing covalently bound protein extracts to CNBr-preactivated Sepharose (Sigma).

Preparation of depletion and affinity columns

Preparation of CNBr-preactivated Sepharose. The resin was washed and swelled in cold 1 mM HCl for at least 30 minutes. A total of 200 ml per gram of dry gel was added in several aliquots. The supernatant was removed by gentle suction in a Büchner funnel between successive additions. The resin was washed with 10 bed volumes of distilled water followed by the additional wash in the coupling buffer, which is 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3-8.5. Five ml of the coupling buffer was used per 1 gram of the dry gel. The resin was immediately transferred to a solution of the appropriate ligand in the coupling buffer.

Coupling of protein extracts to the columns. Three depletion columns were prepared by coupling a) whole bacterial extract containing GST/HOXB5 exon I fusion protein, b) the affinity purified and dialyzed GST and c) GST/HOXB5 exon I fusion proteins to previously prepared resins. 5-10 mg of each protein extract was dissolved in 2 ml of the coupling buffer and used per 1ml of the swelled CNBr-preactivated Sepharose. The protein

extracts were separately incubated with the resin for 2 hours at room temperature using a rotisserie. Unreacted ligands were washed away using 10 bed volumes of the coupling buffer. This wash and subsequent steps were accomplished using the Buchner funnel method. The supernatant was collected to determine a coupling efficiency. Unreacted groups were blocked with 10 bed volumes of 0.2 M glycine, pH 8.0 for 2 hours at room temperature. The blocking solution was removed by extensive washing with 10 bed volumes of the (basic) coupling buffer followed by a wash with 10 bed volumes of 0.1 M CH_3COONa buffer, pH 4 containing 0.5 M NaCl. This wash cycle of high and low pH buffer solutions was repeated for a total of five times. The resin was equilibrated in 10 bed volumes of 1X PBS and immediately used for the sera purification. The procedure described above was also used to prepare the affinity column by coupling the affinity purified and dialyzed GST/HOXA5 exon I fusion protein to the resin.

Crude sera purification

Sera were consecutively passed over three depletion columns containing: a) whole bacterial extract comprising GST/HOXB5 exon I fusion protein, b) purified GST/HOXB5 exon I fusion protein and c) purified GST protein followed by affinity purification on a column with covalently bound purified GST/HOXA5 exon I fusion protein. Four ml of thawed sera were first spun at 3,000X g for 30 minutes at 4^0 C. The supernatant was collected, diluted 1:10 in 10mM Tris, pH 7.5 and used for three consecutive depletions. Each gravity flow-through was collected and the final flow-through was applied to the affinity column. The antibodies were first eluted with 10 bed volumes of 100mM glycine, pH 2.5 and collected into tubes containing 1 bed volume of 1M Tris, pH 8.0 for a total of five fractions.

The column was washed with 10 bed volumes of 10mM Tris, pH 8.8 until pH rose to 8.8. Remaining proteins were eluted with 10 bed volumes of 100mM triethylamine, pH 11.5 and collected in 1 bed volume of 1M Tris, pH 8.0 for a total of five fractions. Immunoreactivity of the affinity-purified sera against the HOXA5 exon 1 moiety separated from GST by thrombin digestion was exclusively found in the acid fractions as assayed by western blotting (data not shown). Each column was regenerated as previously described (Harlow and Lane, 1988). Detailed protocols for preparation of depletion and affinity columns and crude sera purification can be found in the notebook V, pages 51-71, and the notebook VI, pages 2-7.

APPENDIX II. HOXA5 protein pattern is concordant with deduced pattern of the 1.8 kb transcript in mesoderm-derived structures affected by the *Hoxa5* mutation

Transcription of the *Hoxa5* gene leads to a complex pattern of multiple mRNAs: short, 1.8, and three larger transcripts 5.0, 9.5 and 11 kb. *Hoxa5* RNA expression was initially detected in mesenchyme of several structures and the central nervous system by *in situ* hybridization (ISH) analysis using probes that recognized all four transcripts (Dony and Gruss, 1987; Gaunt et al., 1990). Differential expression of the *Hoxa5* transcripts during embryogenesis was revealed by ISH analysis using probes that detected either three larger or all four transcripts. Nonetheless, the occurrence of the short transcript can be deduced due to a lack of hybridization signal with a probe detecting larger transcripts only and the presence of hybridization signal with a probe recognizing all four *Hoxa5* transcripts. In addition, the larger transcripts were expressed in more posterior structures with a temporal delay relative to the short transcript (Larochelle et al., 1999). The anterior limit of expression of the larger

transcripts in the prevertebral column was found at prevertebra (pv) 10, while the inferred expression of the short transcript lay between pv3 and pv10 (Aubin et al. 1998; Larochelle et al., 1999). The presence of the short transcript was also deduced in developing scapula, trachea, thyroid region, myenteric plexus of the gut, and lung mesenchyme (Aubin et al. 1997, 1998, 1999; Larochelle et al., 1999; Aubin et al. 2002a; Meunier et al., 2003). Albeit the *Hoxa5* mutation abolished expression of the four wild-type *Hoxa5* transcripts (Jeannotte et al., 1993), some of embryonic regions where the expression of the short transcript is implied, such as the respiratory tract, gut, the cervical region of the skeleton, scapula, and thyroid gland are affected by the mutation (Aubin et al., 1997, 1998, 1999; Larochelle et al., 1999; Aubin et al. 2002a). Therefore, it appears that the short (1.8 kb) transcript could be the functional form of the *Hoxa5* gene.

To correlate the presence of HOXA5 protein to the intricate *Hoxa5* transcriptional pattern, we studied the expression pattern of the HOXA5 protein during embryogenesis and showed that the protein appeared to mirror the inferred expression pattern of the short transcript in the mesoderm-derived structures affected by the *Hoxa5* mutation (Chapter III). Here, we show a detailed description of the anterior-posterior domain of HOXA5 expression in organs with mesodermal origin on sagittal and serial transverse sections at embryonic days (E) 12.5 and E13.5 (Fig. 2, 3).

To substantiate the specificity of HOXA5 antibodies (chapter III; Fig. 1) the immunohistochemical (IHC) analysis of *Hoxa5 -/-* embryos was performed on sagittal sections. No detectable level of HOXA5 immunoreactivity was seen in specimens at E12.5 (Fig. 2A). In contrast, at the same embryonic age in wild-type (+/+) animals, HOXA5 protein was detected between prevertebrae (pv) 3-10 with the most intense expression approximately

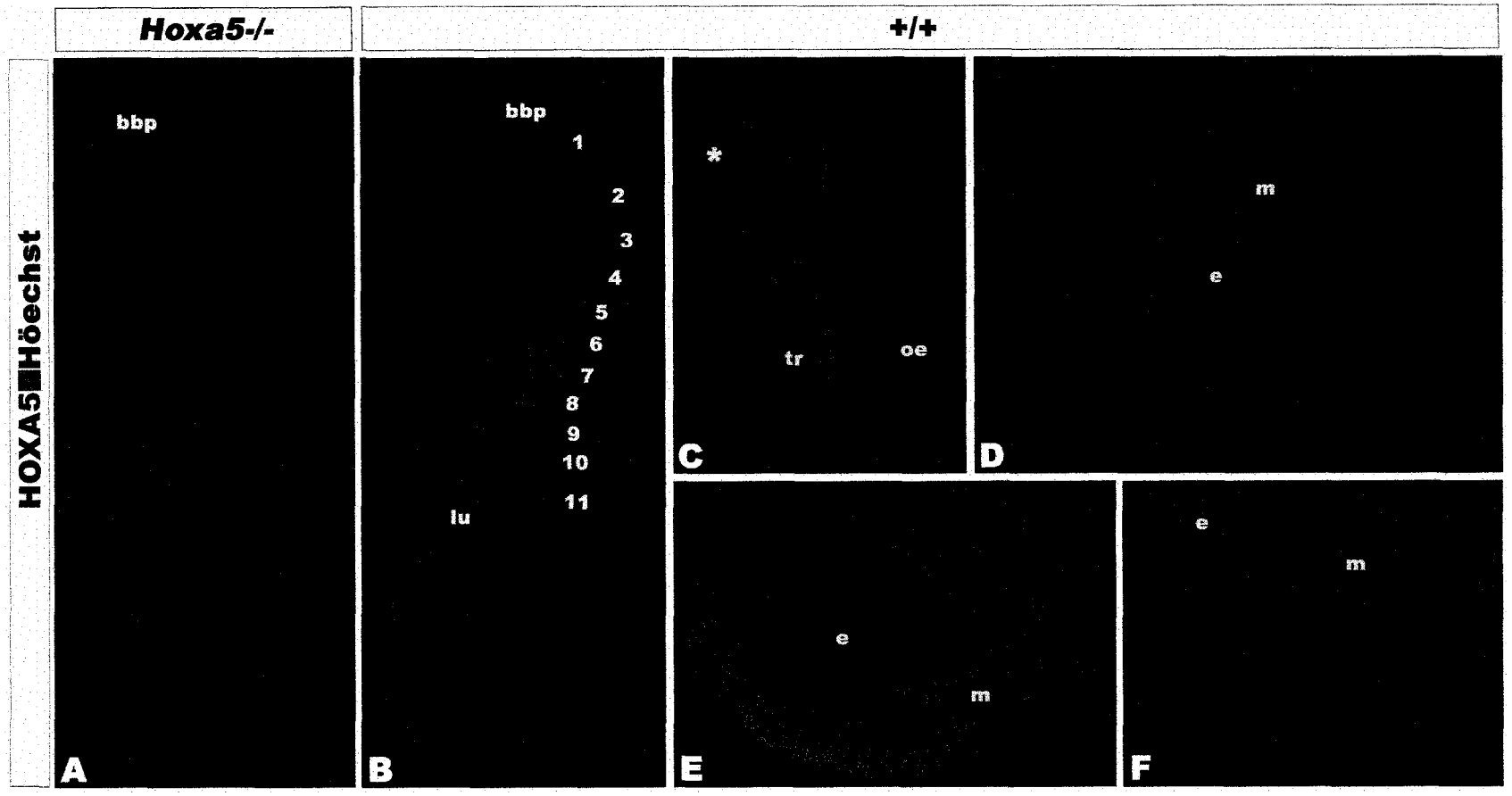

Fig. 2. HOXA5 is expressed in mesoderm-derived structures affected by the *Hoxa5* mutation. (A-F) HOXA5 expression pattern in *Hoxa5* -/- (A) and +/+ (B-F) sagittally sectioned embryos at E12.5. (A) HOXA5 immunoreactivity is absent in *Hoxa5* -/- embryo. (B) HOXA5 expression is confined to prevertebrae (pV) 3-10 along anterior-posterior axis. (C) HOXA5 expression profile in developing trachea (tr). An asterisk indicates HOXA5+ cells in thyroid region. (D, E, F) HOXA5 expression pattern in developing lung (D), stomach (E), and gut (F). The expression is detected in mesenchyme (m) but not in epithelium (e). 1-11, prevertebrae (pV) 1-11, respectively; bbp, basiooccipital bone primordium; lu, lung; oe, oesophagus.

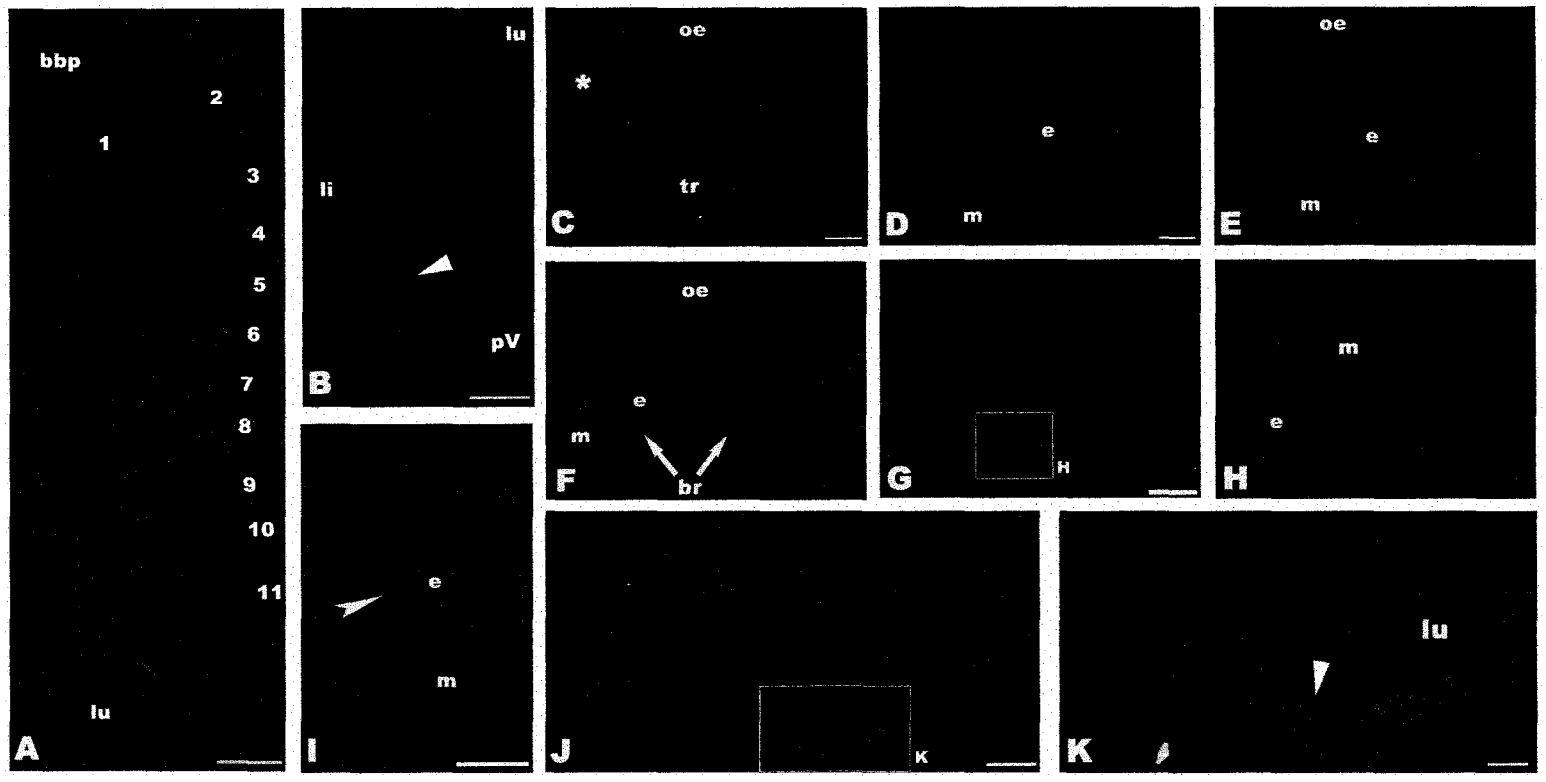

Fig. 3. HOXA5 expression pattern in *Hoxa5* +/+ embryos at E13.5. (A) HOXA5 expression is restricted to prevertebrae (pV) 3-10 along anterior-posterior axis. (B) The expression is detected in developing diaphragm (arrowhead) and lung (lu). (C-E) HOXA5 expression profile in developing trachea (tr) along the anterior-posterior axis. The expression is detected in mesenchyme (m) but not in epithelium (e). An asterisk in C indicates HOXA5+ cells in thyroid region. (F-H, J, K) HOXA5 expression pattern in developing lung along the anterior-posterior axis. The expression is detected in mesenchyme of bronchi (br) and lung but not in epithelia of either organs. H is higher magnification of area indicated by the white-framed box in G. (J, K) The same expression profile is retained in the posterior lung (J). An arrowhead in K indicates HOXA5 expression in developing diaphragm. K is higher magnification of the area indicated by the white-framed box in J. (I) The expression in developing gut (chevron) is confined to the uniquely arranged cell layer within mesenchyme but not in epithelium. Embryos were sectioned sagittally (A, B, and I) and transversely (C-E, F-H, J, and K). 1-11, prevertebrae (pV) 1-11, respectively; bbp, basiooccipital bone primordium; li, liver; oe, oesophagus. Bars (A): 500 μm; (B, C, and I): 100 μm; (D, E, F, H, and K): 50 μm; (G and J): 200 μm.

in the pv 4-9 along anterior-posterior axis (Fig. 2B). In addition, HOXA5-positive cells were found in developing trachea and thyroid region, lung, stomach, and gut but not in oesophagus. Moreover, the expression was detected in mesenchyme but not in epithelium (Fig. 2C-F). At E13.5, HOXA5 expression was retained in pv 3-10 region along anterior-posterior axis (Fig. 3A). The expression was also found in developing lung (Fig. 3B) and diaphragm on sagittal (Fig. 3B) and transverse sections (Fig. 3J, K). Consistent with the expression pattern seen at E12.5, HOXA5 expressing cells were observed in thyroid region and mesenchyme of developing respiratory tract along the anterior-posterior axis (Fig. 3C-K). The expression in gut was also confined to the mesenchyme, in a cell layer that corresponded to developing myenteric plexus of enteric nervous system (Fig. 3I; Aubin et al. 1999).

Our detailed analysis of the HOXA5 expression pattern in mesoderm-derived structures along anterior-posterior axis and developing gut at E12.5-13.5 a) reveals the protein expression in diaphragm, the structure lacking any data regarding the expression of *Hoxa5* transcripts b) further supports a concordance between the HOXA5 protein expression pattern and the specific pattern deduced for the short (1.8 kb) transcript strengthening and confirming the functional significance of this transcript in the development of these structures affected by the *Hoxa5* mutation (Jeannotte et al., 1993; Aubin et al., 1997, 1998, 1999; Larochelle et al., 1999; Aubin et al., 2002a, 2002b; Meunier et al., 2003; Chapter III).

APPENDIX III. Limitation of use of the yeast one-hybrid system to clone Brachial Spinal Cord Enhancer-Binding Protein cDNA

A significant aspect of pattern formation is the transduction of spatial cues to *Hox* target genes resulting in a proper specification of regional identity along the embryonic axis. The transduction is attained primarily through control of *Hox* RNA and/or protein expression by regulators acting at spatial regulatory elements.

Previous studies of the *Hoxa5* proximal promoter defined a *cis*-regulatory element of 604 base pairs termed the brachial spinal cord enhancer (BSCE). This element was capable of directing a gene reporter expression to the C3-T2 region of the dorsal spinal cord, a subset of the *Hoxa5* endogenous pattern (Zakany et al., 1988; Tuggle et al., 1990). Subsequent studies identified a 72 bp region of the BSCE containing three AAATAA motifs named A, B, and C that were, *in vivo,* required for the region-specific expression and bound by individual embryonic proteins (Nowling et al., 1999).

We applied the yeast one-hybrid system (Clontech) in an attempt to directly clone BSCE-Binding Protein (BSCE-BP) cDNAs. Initially, the 72 bp region of the BSCE was used as a bait (ABC bait), as this was critical for protein recognition (Nowling et al., 1999). His+ selection and *LacZ* screening for the ABC motif in yeast transformed with a mouse e11.5 embryo cDNA library were unsuccessful showing a high background and unspecific binding to the mutated ABC bait. Since the three AAATAA motifs differ in their flanking DNA sequences and a mutation of the middle AAATAA motif (B motif) was shown to be the most detrimental for the protein binding to individual motifs (Nowling et al., 1999), we also created a synthetic BBB bait, which in electromobolity shift assay analyses bound mouse

embryonic proteins similarly to the ABC bait. The BBB bait strain had a low background growth, and thus, new selection and screening were attempted. Unfortunately, this new strain, and several other strains obtained from other laboratories, created with the BBB bait, was refractory to further transformation.

In conclusion, the yeast one-hybrid system was not successful in the effort to clone BSCE-BP cDNAs and subsequently identify BSCE-BP. It is feasible that endogenous yeast proteins could bind to the wild-type and mutated ABC bait and that the BBB bait may interfere with the transformation procedure and viability of yeast strains. Future experiments designed to clone and identify BSCE-BP should consider an alternative approach, such as biochemical purification followed by the protein(s) sequencing.

References

Aubin, J., Lemieux, M., Tremblay, M., Bérard, J., Jeannotte, L., 1997. Early postnatal lethality in *Hoxa-5* mutant mice is attributable to respiratory tract defects. Dev. Biol. 192, 432-445.

Aubin, J., Lemieux, M., Tremblay, M., Behringer, R.R., Jeannotte, L., 1998. Transcriptional interferences at the *Hoxa4/Hoxa5* locus: Importance of correct *Hoxa5* expression for the proper specification of the axial skeleton. Dev. Dyn. 212, 141-156.

Aubin, J., Chailler, P., Ménard, D., Jeannotte, L., 1999. Loss of *Hoxa5* gene function in mice perturbs intestinal maturation. Am. J. Physiol. 277, C965–C973.

Aubin, J., Lemieux, M., Moreau, J., Lapointe, J., Jeannotte, L., 2002a. Cooperation of *Hoxa5* and *Pax1* genes during formation of the pectoral girdle. Dev. Biol. 244, 96-113.

Aubin, J., Déry, U., Lemieux, M., Chailler, P., Jeannotte L., 2002b. Stomach regional specification requires *Hoxa5*-driven mesenchymal-epithelial signaling. Development 129, 4075-4087.

Dony, C., Gruss, P., 1987. Specific expression of the *Hox 1.3* homeo box gene in murine embryonic structures originating from or induced by the mesoderm. EMBO J. 6, 2965-2975.

Gaunt, S.J., Coletta, P.L., Pravtcheva, D., Sharpe, P.T., 1990. Mouse *Hox3.4*: Homeobox sequence and embryonic expression patterns compared with other members of the *Hox* gene network. Development 109, 329-339.

Harlow, E., Lane, D., 1988. Antibodies: A laboratory manual, first ed. Cold Spring Harbor Laboratory Press, New York.

Jeannotte, L., Lemieux, M., Charron, J., Poirier, F., Robertson, E.J., 1993. Specification of axial identity in the mouse: role of the *Hoxa-5 (Hox1.3)* gene. Genes Dev. 7, 2085-2096.

Larochelle, C., Tremblay, M., Bernier, D., Aubin, J., Jeannotte, L., 1999. Multiple *cis*-acting regulatory regions are required for the restricted spatio-temporal *Hoxa5* gene expression. Dev. Dyn. 214, 127-140.

Meunier, D., Aubin, J., Jeannotte L., 2003. Perturbed thyroid morphology and transient hypothyroidism symptoms in *Hoxa5* mutant mice. Dev. Dyn. 227, 367-378.

Nowling, T., Zhou, W., Krieger, K. E., Larochelle, C., Nguyen-Huu, M. C., Jeannotte, L., Tuggle, C.K., 1999. *Hoxa5* gene regulation: A gradient of binding activity to a brachial spinal cord element. Dev. Biol. 208, 134-146

Odenwald, W.F., Taylor, C.F., Palmer-Hill, F.J., Friedrich, V.Jr., Tani, M., Lazzarini, R.A., 1987. Expression of a homeo domain protein in noncontact-inhibited cultured cells and postmitotic neurons. Genes Dev. 1, 482-496.

Tuggle, C.K., Zakany, J., Cianetti, L., Peschle, C., Nguyen-Huu, M.C., 1990. Region-specific enhancers near two mammalian homeobox genes define adjacent rostrocaudal domains in the central nervous system. Genes Dev. 4, 180-189.

Wall, N.A., Jones, M., Hogan, B.L.M., Wright, C.V.E., 1992. Expression and modification of Hox 2.1 protein in mouse embryos. Mech. Dev. 37, 111-120.

Zakany, J., Tuggle, C.K., Patel, M., Nguyen-Huu, M.C., 1988. Spatial regulation of homeobox gene fusions in the embryonic central nervous system of transgenic mice. Neuron 1, 679-691.